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CONTENTS

	PAGE
The Relationship of Saprophytic Antagonism to Control of *Botrytis cinerea* Pers. on Tomatoes. *F. J. Newhook*	473
Elimination of Potato Virus Y from a Potato Variety. *A. D. Thomson*	482
Photosensitivity Diseases in New Zealand. IX. *J. V. Evans, D. McFarlane, C. S. W. Reid,* and *D. D. Perrin*	491
A Note on the Incidence of *Ascochyta* Species in Pea Seed. *I. A. M. Cruickshank*	497
Effect of Temperature on Infection by *Pseudomonas syringae* van Hall. *D. W. Dye*	500
A Comparison of Glasshouse and Laboratory Methods for Testing Fungicides against *Botrytis cinerea*. *P. J. Brook*	506
Seedling Studies in Fescue-tussock Grassland. II. *M. J. A. Simpson*	512
Effect of a Permanent Grass Cover on Apple-tree Yield in the First Years after Grassing. *E. G. Bollard*	527
A *Pythium* Disease of Pear Trees. *F. J. Newhook*	533
Crown Rust of Ryegrass. *I. A. M. Cruickshank*	539
Trials of Fruit-setting Sprays on Glasshouse Tomatoes. *R. M. Davison*	544
The Rachitogenic Effect of Vitamin A. *A. B. Grant* and *Patricia B. O'Hara*	548

NOTICES

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THE RELATIONSHIP OF SAPROPHYTIC ANTAGONISM TO CONTROL OF *Botrytis cinerea* Pers. ON TOMATOES

By F. J. NEWHOOK, Plant Diseases Division, Department of Scientific and Industrial Research, Auckland

(Received for publication, 31 October 1956)

Summary

Strains of *Cladosporium herbarum, Penicillium, and Alternaria* isolated from dead tomato petals inoculated in pure and mixed culture on to sterilized dead petals 3–5 days before inoculating with *Botrytis cinerea* gave complete control of the latter fungus.

Establishment of *C. herbarum* and *Penicillium* sp. on dead petals adhering to fruit following use of growth-substance sprays for fruit setting reduced *Botrytis* infection to 1–3%. Infection on trusses of check plants was 46–80%.

Inoculation of dead petals or fruit with dead petals attached, taken from trusses at regular intervals or from trusses of different age, showed that infection rate dropped from almost 100% on newly dead petals to as low as 30% on older petals. Drop in infection rate was correlated with visible growth of saprophytes, mainly *C. herbarum* and *Penicillium* spp.

Infection of leaves with leaf mould (*C. fulvum*) reduced the infection rate of this tissue when dead by *B. cinerea* from 100% to 13–38%.

The nature of the antagonism to *Botrytis* is discussed, particularly in relation to the high level of control by saprophytes such as *C. herbarum* which are not regarded as producers of strongly antibiotic substances.

INTRODUCTION

Although there have been numerous demonstrations of *in vitro* antagonism of plant pathogenic fungi by saprophytic micro-organisms, there have been few *in vivo*. Furthermore, in most of the latter there has been a departure from completely natural conditions in one or more respects, e.g. use of sterilized soil, enrichment of soil with organic amendments, deliberate inoculation of pathogens or saprophytes growing on a quantity of artificial media.

Several cases have been reported where the existence of saprophytic antagonism has been inferred but not proved, e.g. by a comparison of

pathogenic behaviour of certain fungi in sterilized and unsterilized soil. Wood and Tveit (1955), whose broad definition of the term "antagonism" is accepted, cite examples of all the above. A review of the literature, however, showed that the number of conclusive demonstrations that depression of pathogenicity under completely natural conditions has in fact been due to saprophytic activity remains very low. It is significant that there is a virtual absence of these in the complex environment of the soil. What cases there are deal with antagonism on simpler substrates such as tree trunks and branches (Brooks and Moore, 1926; Koch, 1934; Welsh, 1942; Rishbeth, 1950; 1951a, b), wheat stubble and culms (Simmonds, 1947), leaves (Newhook, 1951) and surfaces of seeds (Tveit and Moore, 1954).

The invasion of dead tissue by wound parasites such as *Botrytis cinerea* Pers. is a particularly suitable subject for investigation of antagonism since the substrate supports active saprophytic growth, in contrast to unwounded plant surfaces, and is also less complex than soil; conditions affecting attack are more easily controlled and studied. Newhook (1951) produced evidence which indicated that saprophytic micro-organisms establishing naturally on dead lettuce tissue in the field gave a considerable amount of control of *B. cinerea*. In the present paper the occurrence of similar natural protection against *B. cinerea* on tomatoes is demonstrated, with data indicating the relative importance of the phenomenon and its relation to the use of fungicides, particularly those incorporated in fruit-setting sprays (*vide* Newhook and Davison 1956 a, b, and Davison and Newhook 1956).

EXPERIMENTAL

Strains of *Cladosporium herbarum* Link ex Fr., *Penicillium* sp., and *Alternaria* sp. were isolated from dead petals in commercial tomato houses. In preliminary experiments using the petal-holder method described earlier (Newhook and Davison, 1956a) 100% control of *B. cinerea* was obtained when these fungi were inoculated on to petals and incubated for three days prior to inoculation with *Botrytis* spores. Similar results were obtained using sterilized dead leaf tissue in place of dead petals.

Two experiments were carried out to demonstrate control by *C. herbarum* and *Penicillium* sp. of fruit rot on plants growing in pots in an experimental glasshouse. In each experiment plants were stopped by removing the main shoot one leaf above the second truss. Flowers were set with a spray containing β-naphthoxyacetic acid and, at the first sign of older petals wilting, heavy spore suspensions of the saprophytes were atomized on to the flower trusses.

To prevent growth of air-borne contaminants as much as possible, humidity was kept low in the the experimental units throughout the period during which flowers were set and fruit was swelling. The first spraying of saprophyte spores was to give petals a predominant loading of spores of the test fungi. *C. herbarum* and *Penicillium* sp. spore-suspension sprays were repeated to all trusses when the second-truss flowers were beginning to wilt. When all or most of the petals of the first and second trusses were dead, all trusses except checks were enclosed in plastic bags after being sprayed again with heavy spore suspensions of the saprophytes. When, after seven days incubation, treated trusses were uncovered, it was

observed that petals were almost completely covered with visible growth of the test fungi. At this stage check and treated trusses were sprayed with a heavy suspension of *B. cinerea* spores and all trusses again covered with plastic bags. In addition, glasshouse humidity was kept high. After 9 days the bags were removed and records taken of (a) fruit rot, and (b) sporulation of *B. cinerea* on petals where no fruit rot had developed. In both trials, trusses had been inoculated with saprophytes and *B. cinerea* respectively on the same days but differed from one another in that plants of the second trial were 10 days younger than those of the first. Check petals in the second trial were thus exposed to chance contamination with saprophytes for a shorter period than in the corresponding trusses of the first trial.

TABLE 1. CONTROL OF *B. cinerea* BY PRIOR INOCULATION OF STRAINS OF *Cladosporium herbarum* AND *Penicillium* SP. ON DEAD PETALS ADHERING TO TOMATO FRUITS

Treatment	Total No. Fruit	No. of Rotted Fruit	No. of Fruit with Infection on Petals only	No. of Fruit with No Infection of Petals or Fruit	% Infection
Trial 1					
Check	124	39	18	67	46
C. herbarum	85	0	1	84	1
Penicillium sp.	81	0	1	80	1
Trial 2					
Check	83	55	11	17	80
C. herbarum	90	3	0	87	3
Penicillium sp.	68	2	0	66	3

Results of both trials are presented in Table 1. They show that saprophytic growth on the treated petals gave almost complete control of *Botrytis*. The fact that there were fewer infected fruit on the older check trusses of the first trial than on the check trusses of the second suggests that an effective level of natural saprophytic protection was building up, despite attempts to reduce saprophytic growth by keeping humidity low prior to inoculation with *B. cinerea*.

Three experiments were carried out which indicated how effectively naturally occurring saprophytic antagonism reduced *Botrytis* attack under commercial glasshouse conditions.

1. In a heated glasshouse with an early spring crop, petals were taken from the first truss when the largest fruits were $\frac{1}{2}$–1 in. in diameter. Flowers had been set with a growth-substance spray and dead petals were adhering below the calyx. Petals were mounted on holders as described previously (Newhook and Davison 1956a), dusted immediately with *B. cinerea* spores, and incubated at 21°c. for 10 days. Four weeks later, when first-formed fruits on the fourth truss in the same glasshouse were approximately $\frac{1}{2}$ inch in diameter, petals were

removed from the first and fourth trusses and inoculated as before. After a further three weeks the process was repeated for the first, fourth and sixth trusses. Results are presented in Table 2.

TABLE 2. DEVELOPMENT OF NATURAL PROTECTION BY SAPROPHYTES AGAINST *B. cinerea* INFECTION OF TOMATO PETALS

Petals removed from 1st-, 4th-, and 6th-truss fruit on dates shown and inoculated on petal holders. Petals were newly dead on the youngest trusses sampled on each occasion.

Date	Truss No.	No. of Petals	Growth of *B. cinerea*			% Infection
			* + above + below	** — above + below	Nil	
17 Aug.	1	50	48	0	2	96
15 Sept.	1	80	28	10	42	47.5
	4	80	65	14	1	98.7
9 Oct.	1	50	12	11	27	46
	4	50	22	6	22	56
	6	50	28	16	6	88

* *Botrytis* growth penetrated through petal to agar block on upper surface of petal holder, i.e. equivalent to successful fruit infection.

** No growth on agar block but recognisable growth of *B. cinerea* (conidiophores) seen on petal fixed to lower surface of holder, i.e. growth reduced but possibility of some fruit rot not eliminated.

It will be seen that dead petals from young fruit were almost completely susceptible to infection by *B. cinerea*. Coincident with increased saprophytic growth (mainly of *C. herbarum* and *Penicillium* spp.) resistance of dead petals to infection by *B. cinerea* increased, infection dropping below 50% within 3–4 weeks.

2. From an unheated glasshouse later in the same spring, fruits with adhering petals were removed from the second and sixth trusses when those on the latter were approximately $\frac{1}{2}$ inch in diameter. After stalks had been protected with petroleum jelly, some fruits were inoculated with *B. cinerea* spore suspension. They were incubated in a humidity cabinet for three days (average temperature 18–20°C.) and then removed to a glasshouse unit where the floor was kept wet (temperature range 17–24°C.). Petals were removed from some of the sixth-truss fruit and inoculated as before on petal holders. Petals of the second-truss fruit were too brittle in this case for successful removal and mounting on holders. Results are presented in Table 3.

Fruit-rot and total-infection data for petals on holders agreed closely in the case of the sixth-truss material. *Botrytis* infection on the older fruit was only half that on the younger, which had been exposed to saprophytic contamination for a much shorter period.

Again *C. herbarum* and *Penicillium* spp. were the predominant saprophytic fungi on older dead petals.

TABLE 3. COMPARISON OF SUSCEPTIBILITY OF OLD AND NEWLY DEAD PETALS TO *B. cinerea*, IN RELATION TO FRUIT ROT

Inoculation Method	Truss No.	Total No. of Petals or Fruit	No. of Rotted Fruit	No. of Healthy Fruit	% Infection
Petals on fruit	2	32	12	20	37.5
	6	32	24	8	75
			Growth on Petal Holders		
			** + above + below	** — above + below	Nil
Petals on holders	6*	32	22	3	7	78

* Petals of 2nd trusses too brittle to remove and mount on holders.
** See Table 2, footnote.

3. In an unheated spring-crop glasshouse in which the first three trusses were set with various combinations of fungicide and growth substance, natural infection with *B. cinerea* was too low for trial purposes. One fruit with dead petals adhering below the calyx was removed from each of 75 second trusses in each treatment. After stalks had been protected with petroleum jelly, fruits were sprayed with *B. cinerea* spore suspension and incubated in a humidity cabinet for seven days (average temperature 18–20°C.) before being placed on trays on a glasshouse bench. Infection data and visible saprophytic growth on a relative scale of 0–3 were recorded 20 days later. Results are presented in Table 4.

TABLE 4. RELATIONSHIP BETWEEN INTENSITY OF VISIBLE SAPROPHYTIC GROWTH ON PETALS AND FRUIT AND INFECTION BY *B. cinerea* ON TRUSSES IN A COMMERCIAL GLASSHOUSE

Treatment (Material added to truss spray)	Total No. Fruit	No. of Fruit with *Botrytis* Rot	No. of Fruit with Saprophytic Growth on Petals*			
			0	1	2	3
Check	75	21	0	0	2	73
Dichlone 0.075%	75	9	45	20	10	0
Ferbam 0.42%	73	2	62	8	3	0
Thiram 0.25%	75	8	58	9	7	0

* 0 = Nil
1 = trace
2 = moderate
3 = almost complete or complete cover

All fungicides tested gave a high degree of control of *B. cinerea* and also greatly reduced saprophyte growth compared with the checks. Growth of *C. herbarum* and *Penicillium* spp. was considerable on check

Fig. 1.—Dead petals on check fruit (right) fully colonized by *C. herbarum*, those of thiram-treated flowers (fruit on left) free from saprophytic growth.

petals (Fig. 1) and infection with *Botrytis* was low, as in the second-truss fruit of the previous experiment.

The data from the preceding experiment indicate that control of *B. cinerea* by the use of fungicides on petals is obtained at the expense of losing a considerable amount of the saprophytic control of this fungus. Nevertheless some saprophytic growth, noticeably more *Penicillium* than *Cladosporium*, was still present on some treated petals. Two experiments were carried out therefore to determine the effects of fungicides on growth of *C. herbarum* and *Penicillium* sp. on dead petals. In Trial A, fungicides under test for inclusion with growth substances in combined truss sprays were tested at different concentrations. In Trial B, most fungicides commonly used either in truss sprays or as overall sprays in commercial tomato houses were tested at normal concentrations. Sterilized dead petals on holders were sprayed with fungicide, dried, and dusted with spores of the two test fungi. Results are presented in Table 5.

The results show that while *C. herbarum* is readily controlled, particularly by the organic fungicides used, a strain of *Penicillium* commonly occurring on dead tomato petals in the field was more resistant.

In view of the strongly antagonistic action of *C. herbarum* against *B. cinerea* and because leaf-mould attack on leaves provides considerable areas of dead tissue which could be potential infection sites for *Botrytis*, tests were made to see if *C. fulvum* was similarly antagonistic. In one experiment interveinal segments of leaves severely affected by leaf mould were inoculated with *B. cinerea* spores. Checks consisted of segments from dead portions of leaves of similar age taken from verticillium-wilted plants free from leaf mould. Infection on top (non-sporing) surfaces

TABLE 5. EFFECT OF FUNGICIDES ON GROWTH OF *Cladosporium herbarum* AND *Penicillium* SP. ON TOMATO PETALS

Treatment		*C. herbarum* No. Petals with Growth (max. 5)	*Penicillium* sp. No. Petals with Growth (max. 5)
Trial A			
Check		5	5
Dichlone	0.025	3	5
,,	0.05	5	1 (slight)
,,	0.075	2	2
,,	0.1	3	0
,,	0.15	3 (slight)	2 (slight)
Ferbam	0.14	0	4
,,	0.28	0	5
,,	0.35	0	5
,,	0.42	0	1 (slight)
,,	0.56	0	3 (slight)
,,	0.70	0	2 (slight)
Thiram	0.1	3	5
,,	0.2	0	2 (slight)
,,	0.3	1 (slight)	2 (slight)
,,	0.4	0	0
,,	0.5	0	0

Treatment		*C. herbarum* Growth index 0–4*	*Penicillium* sp. Growth index 0–4*
Trial B			
Check		4	4
Captan	0.2	0	2
Dichlone	0.05	0	2
Ferbam	0.35	0	3
Thiram	0.1	1	3
,,	0.2	0	4
,,	0.3	0	1
Ziram	0.21	4	4
,,	0.42	1	4
Copper oxychloride	0.25	4	3
Cuprous oxide	0.25	3	4
Salicylanilide	0.08	1	2

* Scale 0–4 indicates relative amount of visible saprophytic cover of petals estimated on a total of 10 petals in each treatment.

of leaf-mould-infected leaves was reduced to 38% (checks 100%), bottom surfaces 13%. Thus while infection by *C. fulvum* does not protect leaf tissue completely against *B. cinerea* its antagonistic action is sufficient to reduce considerably the apparent potential risk of *Botrytis* infection offered by large masses of leaf-mould-infected tissue.

DISCUSSION

From the evidence above it is clear that colonization of dead tissue by saprophytic micro-organisms offers a considerable degree of protection against *B. cinerea* under normal glasshouse conditions. The disadvantage, compared with application of fungicides, is that a period of incubation ranging from several days to several weeks is needed for protection to build up to an effective level. The level eventually attained, on dead petals adhering to fruit, is sufficient to reduce losses to below 40% on the first three trusses in houses subjected to severe late *Botrytis* infection. Considerable losses occur however on upper-truss flowers and young fruit.

While the effectiveness of fungicides tends to be reduced with time (e.g. dichlone in glasshouse tests described earlier, Newhook and Davison 1956b) the effectiveness of saprophytic antagonism tends to increase. Fungicides are available however (e.g. ferbam and thiram) which give protection over the whole period between fruit set and harvest.

Fungicides, by reducing the amount of a natural protection mechanism would seem therefore to start off at a disadvantage. In practice, however, if applied correctly, fungicides give a greater degree of protection over the complete danger period for *Botrytis* attack.

Saprophytic antagonism may still play an important part in protecting dead tissue left uncovered by inefficient application of fungicides. The activity of relatively fungicide-resistant saprophytes such as *Penicillium* spp. would of course be additive to the protection offered by fungicides.

The recording of *C. herbarum* as the main natural antagonist to *B. cinerea* on tomato, as on lettuce (Newhook 1951), is of particular interest. Only one other instance has been noted of antagonism by *C. herbarum* against a plant pathogen (against *Pellicularia filamentosa* (Pat.) Rogers on Chinese cabbage, Jaarsveld, 1942). Wood (1951) and Newhook (1951) both demonstrated under controlled conditions that there are many stronger antagonists to *B. cinerea* than *C. herbarum*, some being recognized producers of strongly antibiotic substances; *C. herbarum* however does not fall into this latter class. Moreover the fact that *B. cinerea* is antagonized on dead tissue by almost all of the saprophytic fungi tested (Newhook, 1951; Wood, 1951) suggests that there is some common inhibitive mechanism less specific than that of the normally recognized antibiotic substances. It might well be the same as the almost cosmopolitan mechanism which prevents spore germination when water droplets form on conidiophores, when spores fall back on to the agar surface of a parent colony, or when contaminating spores of all except a few species land on the surface of fully colonized agar plates of either fungi or bacteria.

The antagonism is not, in these cases or in the carefully examined cases of control on inoculated lettuce or tomato tissue, due to lack of moisture, nor is it due to exhaustion of nutrient or growth factors in the substrate, since spores do not even commence germination despite their own reserves of these substances. Accumulation of carbon dioxide or lack of oxygen could only apply in sealed containers, yet the phenomenon may be observed just as readily on exposed colonies or on saprophytically colonized dead plant tissue in the field. Changes in pH of colonized substrates are only partly involved in the antagonism since it occurs on buffered media (Newhook, 1951a).

The factor or factors may be similar to or identical with the unidentified factor causing inhibition of spore germination in soils, described by Dobbs and Hinson (1953). An understanding of the nature of such a ubiquitous and effective factor or factors could lead to important advances in the field of disease control.

Amongst the numerous saprophytes possessing this antagonistic ability, *C. herbarum* and *Penicillium* spp. have the advantage of being able to colonize dead tissue under much drier conditions than most other micro-organisms. It is probably this latter factor more than any other which makes these the commonest and most important protectants of dead tissue against *B. cinerea*.

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ELIMINATION OF POTATO VIRUS Y FROM A POTATO VARIETY

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(Received for publication, 4 September 1956)

Summary

The results of a study on methods of eliminating potato viruses X and Y from selected potato tissue are described and discussed.

Potato virus Y was eliminated from thirteen plants of three strains of the potato variety Aucklander Short Top by keeping sprouted tubers, shoot apex cultures, and stem segment cultures, in the dark at 30°, 35° and 38°C. for up to 23 days and excising new shoot growth. Virus Y was also eliminated from five plants by excising shoots from cultures without prior heat treatment. The highest percentage of virus-Y-free plants was obtained by establishing etiolated shoot apices in culture and then heat treating. Treatments did not eliminate potato virus X from either Aucklander Short Top or Dakota varieties.

The most satisfactory line of virus-Y-free Aucklander Short Top may be one infected with an avirulent virus-X strain.

A preliminary glasshouse experiment showed some increase in yield of plants infected with virus X compared with those infected with X + Y.

Introduction

A reliable method for eliminating potato viruses X and Y from selected tubers would be of considerable importance. Norris (1954) and Morel and Martin (1955) have reported the elimination of virus X from potatoes, and the latter have also reported the elimination of virus Y.

The potato varieties Dakota and Aucklander Short Top were chosen for the present work. Dakota is infected with virus X and Aucklander Short Top is normally infected with viruses X and Y (Procter, 1952). Despite the high incidence of virus infection, these varieties are the most commonly grown in New Zealand.

Review of Literature

Recent investigations into methods of eliminating potato viruses have been concerned with the use of plant-virus inhibitors in tissue cultures, and heat treatment of tubers.

Plant-virus Inhibitors

A single virus-X-free plant of the potato variety Green Mountain was obtained by Norris (1954) from shoot-apex cultures with malachite green added to the culture media. Virus X was not eliminated from Dakota nor viruses X and Y from Aucklander Short Top by this method (Thomson, 1956a). Preliminary experiments using the pyrimidine base analogue thiouracil, with a similar tissue-culture technique were also unsuccessful (Thomson, 1956a).

The failure of these techniques was possibly due to the inability of the inhibitors to penetrate to all sites of virus formation. Basic dyes

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have been shown to be less readily absorbed by plant material than acidic dyes and a possible explanation has been suggested by Charles (1953).

Stoddard (1947) and Holmes (1955a) appear to be the only workers who have made some practical use of a diverse group of chemicals known to affect plant viruses. At present other methods appear more likely to free vegetatively propagated plants from virus.

Heat Treatment

Kassanis (1949) and Kunkel (1943) have eliminated leafroll virus and witches'-broom virus respectively by heat treating potato tubers. Although there have been no reports of tubers being freed from viruses X and Y by heat treatment, high temperatures are known to reduce the severity of potato-mosaic symptoms (e.g., Johnson, 1922). This "masking" effect appears to be associated with low virus content in the case of virus X (Pound and Helms, 1955).

Sheffield (1942) has shown that tobacco-mosaic virus systemically invades the apical meristem of tobacco. However, recent studies with other viruses and hosts indicate that systemic invasion does not include the apical region of the shoot, and by excising this region virus-free plants can be developed. This technique has been applied to dahlias infected with dahlia-mosaic virus (Morel and Martin, 1952) and tomato spotted-wilt virus (Holmes, 1955b), and to sweet potato infected with internal-cork disease (Holmes, 1955c). The recent report of Morel and Martin (1955) indicates that viruses X, Y, and A can also be eliminated by excising the apical meristem of potatoes.

It should be possible to obtain virus-free plants by the combination of a method that will reduce the virus concentration or delay its systemic spread without completely inhibiting the growth of the host plant, followed by the excision of the new plant growth. This technique would not necessitate the removal of the small apical meristematic region. Thomson (1956b) successfully used such a combination of heat treatment with tissue culture to obtain plants of Aucklander Short Top free from virus Y. The following is a complete report on this work.

EXPERIMENTAL METHODS

Three heat-treatment and tissue-culture methods were used:

1. Heat treatment of tubers that had been sprouted in darkness, and excision of sprouts after treatment.
2. Heat treatment of apex cultures from etiolated shoots, and excision of new apical growth.
3. Heat treatment of stem-segment cultures, and excision of new central axillary-shoot growth.

Shoots were also excised from sprouted tubers, shoot-apex cultures, and stem-segment cultures maintained at room temperature.

The culture media used and the methods of tissue culture and of testing plants have already been described (Thomson, 1956b). All cultures were maintained in the dark in incubators at room temperature, 27°, 30°, 35°, 37°, and 38°C. Excised shoots were initially grown in culture in a glasshouse before being transferred to soil in pots. The length of

excised tissue varied from 0.5 to 2 cm. depending on the amount of new growth.

All tubers and plants used in the various experiments were tested for the presence of virus prior to being used, and all Aucklander Short Top material was found to be infected with viruses X and Y and all Dakota with virus X.

Fig. 1.—Growth of axillary shoots from the nodes of stem segments: left to right, two cultures each of Aucklander Short Top segments kept at 37°, 30°, and 27°C. for 14 days. Slight tuberization, small leaves, and long internodes at 30° and 27°C.

Photo: C. J. Miles.

Stem segments (Fig. 1) were used in the hope that axillary shoot growth would be more rapid (Mes and Menge, 1954). Stem segments, each including one node, were cut from defoliated plantlets grown from potato shoots.

Results

Table 1 is a summary of the experiments that were successful in eliminating virus Y. No virus-X-free plants were obtained from any of the experiments.

A total of 81 heat-treated Aucklander Short Top plantlets survived for testing, and 13 of these were free from virus Y (Figs. 2 and 3). A total of 65 untreated plantlets survived (all from shoot-apex cultures) and 5 of these were free from virus Y. All three heat-treatment methods yielded plants free from virus Y. The percentages of virus-free plants amongst the survivors of heat-treated sprouted tubers, shoot apices, and stem segments were 15, 36, and 6 respectively. The establishment of shoot apices in culture and their subsequent transfer to incubators kept at 35°C. for up to 2 weeks appears to be the most satisfactory method for

TABLE 1. ELIMINATION OF POTATO VIRUS Y FROM AUCKLANDER SHORT TOP

Material Heat-treated	Strain	Start of Exp.	Treatment	No. of Apices Detached†	No. of Cultures Surviving	No. Free From Virus Y	No. of Tests‡
Shoot cultures	Redmond	6.8.54*	35°C: 17 days	2	1	1	3 + 1
Shoot cultures	Redmond	14.2.55*	35°C: 16 days	9	7	5	2 + 1
Sprouted tuber	Redmond	3.12.54	35°C: 7 days	10	2	1	2 + 1
Sprouted tuber	Redmond	9.2.55	38°C: 7 days	12	9	4	2 + 1
Stem segment cultures	Oakley	10.10.55*	30°C: 23 days	5	5	1	1
Stem segment cultures	Jarman B.	9.1.56	35°C: 15 days	5	3	1	1
Shoot cultures	Redmond	14.2.55*	Room temperature: 16 days (13-26°C.)	4	3	3	1 + 1
Shoot cultures	Redmond	9.2.55*	Room temperature: 45 days (13-26°C.)	9	1	1	1
Shoot cultures	Redmond	13.4.55*	Room temperature: 38 days (12-25°C.)	7	7	1	1

* Cultures maintained for 1-2 weeks in glasshouse before transferring to incubators.

† One apex (0.5-2 cm) detached from each culture.

‡ First figure indicates the number of tests on plantlet and second figure the number of tests on plants regrown from tubers.

Fig. 2.—Two Aucklander Short Top plants infected with virus X (left) and two with viruses X + Y (right). All derived from the same tuber. Photo taken in winter.

Photo: C. J. Miles.

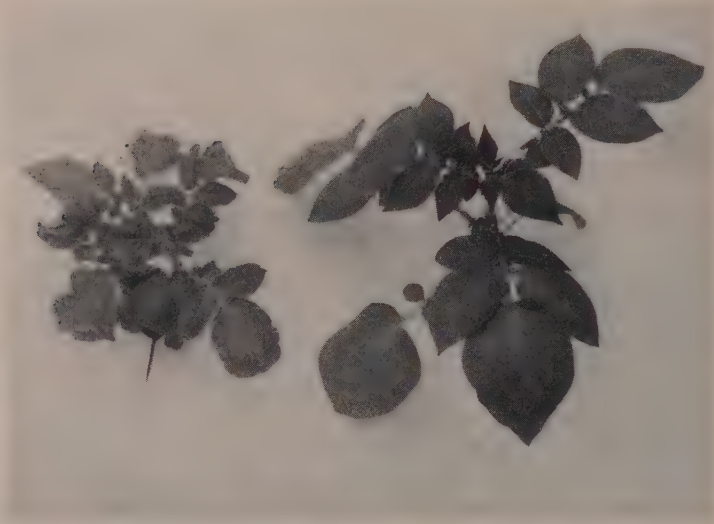

Fig. 3.—Aucklander Short Top plants infected with viruses X + Y (left) and with virus X (right). Both were grown from tubers, and were originally derived from the same tuber. Photo taken in summer.

Photo: C. J. Miles.

eliminating virus Y. The lower percentage of virus-free plants from stem segments was probably due to a higher initial virus concentration in the stems compared with that in etiolated potato shoots.

At the higher temperatures used (37° and 38°c.) shoot growth was slow and many cultures died. At other temperatures the shoot growth was rapid.

Similar treatments did not yield virus-X-free Dakota plantlets amongst the 38 that survived for testing.

Heat-treated tubers and the remaining portions of cultures after shoot excision were planted and, although the majority failed to grow, those that did proved to be infected with virus X (Dakota) and virus X + Y (Aucklander Short Top).

Virus Y attenuation in the treated plantlets could have been responsible for the more vigorous plant growth, but cross-protection studies with a severe strain of virus Y on the test plants was always negative; virus attenuation was therefore not the reason. Furthermore, virus Y was not merely reduced in concentration, rather than eliminated, for 14 of the original treated plantlets were regrown from tubers and virus Y was not recovered from them.

Tubers were formed only by stem-segment cultures, and these were formed at temperatures of 30°c. or lower (Fig. 1). Mes and Menge (1954) have clearly described the ways in which tuberization takes place in culture with axillary shoot growth from stem segments. In the present work all the peculiar growth habits described by Mes and

Menge were observed. Root growth was observed in all cultures except stem-segment cultures kept at temperatures of 35°, 37°, and 38°C.

Strains of Aucklander Short Top

A number of strains of Aucklander Short Top are known to exist and some of these strains have consistently outyielded others in experiments conducted by the Department of Agriculture (unpublished data). In the experiments reported here, the highest-yielding strain (Redmond) was used most frequently but virus Y was eliminated also from two other strains, namely Oakley and Jarman B (Table 1).

Virus X in Aucklander Short Top

Thomson (in press) indicated that the variety Aucklander Short Top is normally infected with a mixture of mild and severe strains of virus X. An avirulent strain of virus X (AST4) has been noted in a number of plants.

The avirulent strain in combination with virus Y under glasshouse conditions produces milder symptoms than the "normal" virus X + Y. This strain may protect plants in the field from more severe strains, although it is unlikely that protection will be complete (Bald, 1948; Best, 1954). Attempts are being made to eliminate virus Y from Redmond plants infected with avirulent virus X.

Yield of Plants Free from Virus Y

In a small-scale glasshouse experiment during 1955–56, the yield of plants infected with virus X was compared with the yield of plants infected with viruses X + Y. All five lines were from the Redmond strain. Accurate comparison is valid only between plants of line E infected with viruses X + Y and those infected with virus X, both of which originated from the same tuber. All tubers were subjected to similar treatment before planting.

TABLE 2. YIELDS OF AUCKLANDER SHORT TOP PLANTS (REDMOND STRAIN) INFECTED WITH VIRUS X AND WITH X + Y

Line	Virus Content	No. of Plants	Mean No. of Tubers per Plant	Mean Tuber Yield per Plant (g.)	Percentage Yield Increase Compared with E (X + Y)
A	X	10	2.1	34.4	31.3
B	X	10	2.1	32.8	25.2
C	X	10	1.5	29.2	11.5
D	X + Y (avirulent virus X)	10	2.1	27.4	4.6
E	X	9	2.4	28.6	9.2
E	X + Y (normal virus X)	13	1.6	26.2	—

Table 2 indicates the results of this experiment. Yields from all plants were small, although all lines free from virus Y outyielded those infected with viruses X + Y. The difference in yield between plants of line E infected with virus X and those infected with X + Y was significant at the 5% level of probability.

Discussion and Conclusions

Plant viruses with low thermal-inactivation points *in vitro* may be directly inactivated *in vivo* by heat treatment. Kassanis (1954) has shown however that viruses with comparatively high thermal-inactivation points can be eliminated by heat treatment. The work of Pound and his associates (e.g. Pound and Weathers, 1953) on the effect of different temperatures on virus concentration in growing plants has emphasized the importance of the host-virus complex. In the present work it is considered that heat treatment may be acting either through inhibiting some cell process necessary for virus synthesis or through delaying the systemic spread of virus Y. Both of these factors may be involved.

The work presented shows that plants free from virus Y can be obtained simply by excision of the apical region of etiolated potato shoots. That a few virus-free plants would be obtained in this way is indicated by the work of Valleau and Johnson (1930) who had difficulty in recovering virus Y from etiolated potato-shoot apices. Stapp and Bartels (1950) have shown also that there is a progressive reduction in virus-X concentration from the shoot base to the apex. There may be a similar reduction in virus-Y concentration, but this has not been investigated.

It is unlikely that either attenuation or reduction in concentration of virus Y, without elimination, was the cause of the increased vigour of the plants as is shown by cross-protection studies and further tests on plants regrown from tubers. The plants are therefore regarded as completely free from virus Y.

No plant has yet been obtained free from virus X. However, continued heat treatment and subculture may reduce the virus-X concentration and it may be possible to free a shoot from this virus. The technique of Morel and Martin (1955) may also provide a method of eliminating virus X. If plants completely free from virus X cannot be obtained, plants free from virus Y and infected with an avirulent strain of virus X should be the most satisfactory, provided they have the required agronomic characters.

Bawden, Kassanis, and Roberts (1948) and Norris (1953) have emphasized the necessity of judging virus-free replacement clones on agronomic characers before they are widely grown. With this in view, virus Y has been eliminated from plants of the Oakley and Jarman B strains as well as the Redmond strain of Aucklander Short Top. Further experiments will be necessary to determine the comparative yield under field conditions of plants free from virus Y and those infected with it. However, replacement of plants infected with virus X + Y will remove an important reservoir of virus Y.

It may be possible to use the technique described here to eliminate viruses from a number of different vegetatively propagated plants. The observations that after heat treatment virus reinvades healthy tissue

(Kunkel, 1943; Kassanis, 1954) indicates that, by applying the subculture method, virus could be completely eliminated.

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PHOTOSENSITIVITY DISEASES IN NEW ZEALAND

IX. THE SUSCEPTIBILITY OF THE GUINEA PIG TO FACIAL ECZEMA

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Summary

A sample of dried pasture which produced facial-eczema-like liver damage when fed to a sheep, consistently caused liver lesions of a similar nature in guinea pigs.

The histopathological lesions in affected guinea-pig livers are described.

Introduction

Facial eczema, an economically important disease of sheep in New Zealand, has been described by Cunningham, Hopkirk, and Filmer[4]. Characteristically, the disease occurs in grazing sheep in the autumn. Affected animals invariably show a severe obliterative cholangitis with subsequent bile ductule hyperplasia, fibrosis, and atrophy of the liver parenchyma. These changes are often associated with impaired excretion of phylloerythrin and bilirubin, leading to photosensitization and jaundice in the affected animals. Field observations extending over many years indicate that the liver damage is due to a toxic substance present in certain types of pasture growing under abnormal climatic conditions[2, 6, 7].

The collection of toxic pasture for experimental use has presented a difficult problem. Outbreaks of facial eczema cannot be accurately forecast. Further, owing to the time lapse between ingestion of the toxic feed by the sheep and the development of identifiable liver lesions or clinical signs, pasture collected during an outbreak is very frequently no longer toxic. A method has, therefore, been evolved of systematically cutting and rapidly drying grass from selected paddocks during late summer and autumn. Sheep grazing the sampled pasture are serially killed and replaced and the livers of the killed animals examined histopathologically. From the occurrence of lesions in these livers it is possible to delimit broadly any periods of pasture toxicity[5]. The toxicity of samples cut and preserved during these periods may then be confirmed in sheep by pen-feeding, slaughter, and subsequent examination of the livers. By this rather complex process, samples of dried pasture of verified toxicity have been obtained.

Hitherto the only test animal for facial-eczema investigations has been the sheep. The almost insuperable practical problems presented in collecting and handling sufficient toxic material for experiments using this

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animal have been one of the major factors limiting progress, particularly in the chemical fractionation of toxic grass. The importance of finding a suitable small animal has been recognized from the outset. Several different species, including the guinea pig, have therefore been tested in the past, but none was considered satisfactory. The guinea pig was found to be susceptible to the facial-eczema toxin but not particularly sensitive to it. However, our experience with this animal in the histopathological investigation of *Panicum miliaceum* poisoning and other diseases associated with photosensitization consequent on liver damage suggested the desirability of re-examining it as a test animal in facial-eczema research. This was made possible when a sample of dried pasture of moderate toxicity became available.

Using the method of serial killing outlined above, characteristic facial-eczema liver lesions were found in lambs grazing a field at the Manutuke Research Station during the period 9 to 17 April 1951. When a total of 38 kg. of dried grass, cut from this field during this time, was fed over 41 days to a sheep of bodyweight approximately 25 kg., characteristic liver lesions were produced. The remainder of this sample of toxic pasture, predominantly short-rotation ryegrass[3], was used in the experiments reported in this paper.

Experimental

The following experiments were carried out:

1. Six guinea pigs were fed toxic dried grass for 21 days and were then killed.
2. Fourteen guinea pigs were fed toxic grass and died or were killed after feeding periods lasting up to 50 days.
3. Ten guinea pigs were fed toxic pasture, three being killed after 14 days feeding, two after 21 days, three after 28 days and two after 42 days.
4. As a control experiment, seven guinea pigs were fed on a sample of normal (apparently non-toxic) dried Manutuke pasture cut on 20 March 1950, two animals being killed after 14 days, three after 28 days, and two after 42 days. In addition, three further pasture samples dried at Manutuke were fed to guinea pigs (two per sample) which were killed after 28 days.

In all cases, guinea pigs of 200–300 g. bodyweight were used. Experiments 1 and 2 were carried out at the Wallaceville Animal Research Station, using indoor-bred animals fed a synthetic diet supplemented with green feed. In experiments 3 and 4, carried out at the Ruakura Animal Research Station, outdoor-bred animals grazing pasture and given supplements of a mash containing milk powder, meat meal, barley meal, bran, and salt, were used. All animals were transferred to individual cages. Those at Wallaceville were housed in a heated, darkened room, while those at Ruakura were in an unheated, non-darkened room. At no time during any experiment were the animals exposed to bright sunlight.

In all experiments, the material fed was given *ad lib.* The animals fed toxic grass ate 10–30 g. daily. The average daily intake was higher in experiment 3 (30 g.) than in experiments 1 and 2. Water was supplied *ad lib.* to all animals.

All animals were weighed daily in experiment 1, weekly in experiment 2, and at two-day intervals in experiments 3 and 4. Except for the 42-day animals, all guinea pigs fed dried toxic grass in experiment 3 showed a steady increase in bodyweight throughout the experiment, while those in experiments 1 and 2 tended to lose weight.

All animals were examined daily for signs of jaundice. Blood samples were taken by cardiac puncture at frequent intervals in experiments 3 and 4 and were examined for bilirubinaemia using the Van den Bergh test. All animals surviving on the 28th day in experiment 2 were similarly examined.

Phylloerythrin estimations could not be carried out on plasma in these experiments as no suitable optical cells for quantitative microspectrophotometry were available to us at the time.

A post-mortem examination was carried out on all animals. Liver slices were fixed in formal calcium[1] and sections examined histologically.

RESULTS

Macroscopic Appearance of Affected Livers

With the exception of the animals killed at 14 days, all livers from guinea pigs fed on the dried toxic grass showed macroscopic lesions. In most cases, the liver was slightly enlarged with the edges of each lobe slightly thickened and rounded.

Under the capsule and on the cut surface two main features were visible: (1) Small (0.5–2.0 mm. diameter) spherical, dull white, opaque, and lustreless foci. (2) Regional exaggeration of the normal portal tract pattern.

Histopathology

No significant abnormality was observed in livers from guinea pigs killed at the end of 14 days feeding on dried toxic grass. In animals killed after 21 days feeding, specific lesions were found, the most characteristic feature being a slowly spreading hyaline coagulative necrosis of the parenchymal cells. In contrast, animals killed after 28 to 50 days feeding showed progressive cholangio-obstructive changes associated with massive ductule proliferation.

Parenchyma

In animals killed after 21 days feeding, the parenchymal tissue was greatly reduced by hyaline coagulative necrosis and in more advanced cases by the invasion of proliferating ductule epithelial cells. The necrosis appeared to spread slowly and to cause a mild macrophage reaction. In some cases the necrosis was associated with haemorrhage.

The site of the necrosis was not constant, for in some cases a small portal tract was involved, while in others no tractal elements could be seen in the necrotic area. The extent of the necrosis varied from cases where a few cells only were involved, to cases where two or three lobules had become necrotic. In these large areas of necrosis the nuclei of the portal tract epithelial cells and the reticuloendothelial cells often retained their ability to stain with basic stains. The necrotic areas were often sharply demarcated from the adjacent relatively normal parenchymal

cells. They appeared to be non-progressive lesions since the number of necrotic areas was greatly reduced in livers of animals killed after 50 days feeding, and those present were resolving. All stages of resolution were seen.

The parenchymal cells—apart from areas of necrosis—showed hypertrophy, considerable variation in size, shape, and staining characteristics of the nuclei and cytoplasm and a general loss of cell outlines. These changes were more marked periportally. In other instances in an area that had partly lost its affinity for acidophilic stains, individual parenchymal cells might be intensely eosinophilic and shrunken, and contain a pyknotic nucleus.

Portal Tracts

In animals killed at 21 days the portal-tract lesions were not marked, but there was hypertrophy and mild hyperplasia of duct epithelium. Venous thrombi were occasionally seen, and in both 21- and 50-day lesions the hepatic arteries were constricted. In some cases, the portal tracts were involved in the hyaline necrosis of the parenchyma described above.

In many instances, in medium and large ducts the epithelial cells tended to lose their affinity for both acid and basic stains. They were hypertrophic, sometimes elongate with ragged margins, and contained a pale nucleus. These changes ranged from the above to a complete replacement of both large and small ducts by mature dense fibrous tissue.

Both large and small tracts in livers from guinea pigs fed dried toxic pasture for 50 days showed marked bile-ductule hyperplasia. In the large tracts, the proliferating tissue radiated out in long cords into the parenchyma from the periphery of the tract, but did not invade the connective tissue surrounding the tractal elements. In the smaller tracts, the original ducts and ductules and associated elements were often indistinguishable among a mass of unorganized proliferating epithelial cells.

Prominent dilated lymphatics and oedema of the connective tissue gave the medium and large portal tracts an open texture even in cases where ductule proliferation was not a marked feature.

The small ducts and ductules associated with major ducts in severe cases often showed an intense basophilia due to mucus accumulation. All stages were seen from ductules with a few basophilic granules within the cytoplasm of some hypertrophic cells, to cases where the outline of the ductule had been completely lost and its place taken by an intensely basophilic, apparently structureless mass. An occasional basophilic goblet cell was seen in the lining cells of ducts associated with these intensely basophilic ductules.

Controls

No gross lesions were observed in the 14- or 28-day control animals. One small focus of necrosis was seen in one liver lobe from one of the two animals that had been fed for 42 days on the normal Manutuke pasture cut on 20 March 1950.

Bilirubin Determinations

Bilirubinaemia was not detected in any of the guinea pigs tested.

Discussion

The object of this investigation was to determine whether the guinea pig is a possible experimental animal for facial-eczema research. In order to do this, it must be shown that grass known to produce the disease in sheep is toxic to the guinea pig, that the lesions produced are sufficiently specific for accurate diagnosis, and that the animal is sufficiently sensitive to the toxin for practical use.

Feeding experiments carried out at Wallaceville in 1943, using dried grass collected in Wairoa and Gisborne during dangerous periods, showed that grass toxic to sheep was also toxic to guinea pigs (Swan, unpublished results). This finding was confirmed in the present work at Ruakura. The guinea pig is clearly susceptible to toxic grass.

The cholangio-obstructive biliary hyperplastic lesions that were found in the guinea pig in the present experiments resemble very closely the lesions seen in sheep becoming affected over an equivalent period. The necrotic lesions, however, are not found in the sheep. Necrotic lesions were a prominent feature of the histopathology of the livers of guinea pigs fed toxic grass in 1943. In contrast to the present findings, these lesions appeared to persist, in one case eight out of ten animals showing severe necrosis of the liver after 49 days feeding. In three of these animals, severe cholangio-obstructive lesions were present as well as severe necrotic lesions. Although the relationship between the two types of lesion is not apparent, the characteristic appearance of the necrotic lesions, and their constant occurrence, indicate that they are an essential feature of the disease in the guinea pig. It is clear, therefore, that the lesions produced in this animal are specific and allow accurate diagnosis.

The absence of hyperbilirubinaemia in the guinea pig parallels the common finding in naturally occurring cases of facial eczema in sheep where the liver lesions have developed slowly over a four to six week period. Although the liver lesions may be quite severe in such cases they appear to have developed sufficiently slowly to allow functional compensation to take place. No attempt was made to detect photosensitivity in the present investigation.

A rough measure of the sensitivity of an animal to a toxin is given by the intake per unit bodyweight required to produce a particular effect. It is possible to compare the sensitivities of the sheep and the guinea pig to the facial-eczema toxin on this basis, using the production of the cholangio-obstructive lesions as a reference. In both the 1943 experiments and those reported here, when the same material was fed to the two species, the guinea pig appeared to be somewhat less sensitive than the sheep. The data available are insufficient to permit any firm conclusions. Nevertheless it is clear that the guinea pig is sensitive enough for normal use as a test animal. As in the case of sheep, variation in the sensitivity of individual guinea pigs has been observed.

The guinea pig thus appears to be a suitable animal for use in facial-eczema research. This has been confirmed using procedures subsequently developed at Ruakura for testing toxic grass samples and extracts.

The savings in the quantity of toxic material used in an individual experiment are substantial when the guinea pig is the test subject. Only

$^1/_{10}$th to $^1/_{20}$th quantity necessary for a sheep-feeding experiment was required for a guinea pig in the 1943 experiments, and approximately $^1/_{50}$th in the experiments recorded here. These differences in proportion are a reflection of the sizes of the guinea pigs used. In 1943, animals averaging 650 g. eating 80–100 g. of dried grass per day were used, while the present animals were 200–300 g. and ate only about 30 g. daily.

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A NOTE ON THE INCIDENCE OF *Ascochyta* SPECIES IN PEA SEED

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Summary

Results of a survey of the incidence of *Ascochyta* spp. in pea seed from Canterbury and Marlborough over four years are presented.

Mycosphaerella pinodes was shown to be the most common of the three seed-borne fungal pathogens of the collar-rot complex.

The importance of dry weather conditions for the production of disease-free seed is confirmed.

Introduction

The importance of sowing disease-free seed is well established and has recently been ably discussed by Noble (1951). *Ascochyta pisi* Lib., *A. pinodella* Jones, and *Mycosphaerella pinodes* (Berk. et Blox.) Niessl (syn. *A. pinodes*), which form the collar-rot complex, are major diseases of peas in New Zealand (Cruickshank, 1951). The prevalence and significance of pea-seed infection by *Ascochyta* spp. has been reported by Crosier (1936), Hickman (1951), and Skolko *et al.* (1954). The present note reports the results of a seed-disease survey over a four-year period in New Zealand.

Materials and Methods

An annual seed-disease survey was initiated in 1951. Half-pound lots of seed peas were obtained for testing from representative crops of the main varieties Greenfeast, William Massey, and Onward, from Canterbury and Marlborough in 1950–51 and 1951–52. In 1952–53 and 1954–55 seed lots from Canterbury only were tested. The seed lots were subsampled by the method of quartering to obtain a final sample of 100 seeds. Triplicate samples of each seed lot were tested.

The agar-plate method of examination was used. Ten subsamples, each of 10 seeds, were wrapped in muslin squares, dipped in 70% aqueous ethanol, steeped in full strength 'Nados'† (10 min.), rinsed in sterile water, and drained on circular discs of Whatman seed-test paper. The seed was plated on to 'Difco-prune' agar (10 seeds per plate) and incubated at 20°C. for 4 days. Subcultures were transferred to potato dextrose agar or oatmeal agar slopes and incubated at 20°C. prior to identification (Jones, 1927).

Results

During the period 1950–51 to 1954–55, 175 seed lots were examined in triplicate and 716 cultures of *Ascochyta* spp. isolated and identified. The percentage of infection in pea-seed lots each year was grouped into

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† Nados (2.25% NaClO in 15.0% NaCl. Manufactured by H. F. Stevens Ltd., Christchurch.

infection classes viz. 0, <1, 1–5, 5–10 and >10%. The percentage of seed lots in each infection class is presented in Table 1. A comparison of the incidence of the three *Ascochyta* spp. isolated from the pea seeds examined is presented in Table 2.

TABLE 1. PERCENTAGE OF *Ascochyta* INFECTION IN PEA-SEED LOTS (mean of three samples of 100 seeds)

Rainfall (inches) Jan.-Mar.	Year	District	Percentage of Seed Lots in each Infection Class					Total No. of Seed Lots
			0	<1	1-5	5-10	>10	
12.9	1950-51	Canterbury	12.5	25.0	50.0	6.2	6.2	16
10.1	1952-53	Canterbury	6.2	10.4	64.6	10.4	8.3	48
8.2	1950-51	Marlborough	60.0	20.0	20.0			10
4.0	1954-55	Canterbury	78.3	17.4	4.3			23
3.8	1951-52	Marlborough	52.1	39.1	8.6			23
2.1	1951-52	Canterbury	65.4	23.8	12.7			55

TABLE 2. INCIDENCE OF *Ascochyta pisi*, *A. pinodella*, AND *Mycosphaerella pinodes* IN SEED SAMPLES

Year	Percentage of Cultures Identified			Total No. of Cultures
	A. pisi	*A. pinodella*	*M. pinodes*	
1950/51	24.6	8.5	66.9	130
1951/52	26.8	18.3	54.9	71
1952/53	11.8	0.6	87.6	508
1954/55	—	14.3	85.7	7

DISCUSSION AND CONCLUSIONS

Surface-sterilizing seed eliminates many surface-borne fungi; however, as seed treatment (organo-mercurial or thiram dusts) of peas is generally practised in this country, only internally seed-borne pathogens are of economic significance. The rainfall records (*New Zealand Gazette, 1950–55*) for January to March (Table 1) indicate that in general the summers of 1950–51 and 1952–53 were wet (rainfall > 8 in.) while those of 1951–52 and 1954–55 were dry (rainfall 4 in. or less). The correlation between rainfall and the angular transformation of the percentage of seed lots with less than 1% infected seeds was 0.843, significant at $P < 0.05$. Thus the level of *Ascochyta* infection in the two wet seasons was significantly higher than that in the drier seasons.

A. pisi in earlier reports (Hickman, 1951; Skolko *et al.*, 1954) has shown to be the most common of the three *Ascochyta* species infecting pea seed. In this country *M. pinodes* appears to be the most

common species (Table 2). This result confirms field-survey observations reported by Brien *et al.* (1955).

From the results of this survey it is concluded that *Ascochyta* spp., especially *M. pinodes,* are common seed-borne pathogens in New Zealand-grown peas. The incidence of disease, however, varies among individual seed lots within a season. Similarly the general level of disease incidence varies significantly from season to season. The importance of low-rainfall conditions for the production of disease-free seed is confirmed.

ACKNOWLEDGEMENTS

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THE EFFECT OF TEMPERATURE ON INFECTION BY *Pseudomonas syringae* Van Hall.

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(*Received for publication, 28 August 1956*)

Summary

Experiments have been carried out at temperatures ranging from 35.5° to 82°F. (1.9° to 27.7°C.) to determine the optimum temperature for infection of peach trees with *Pseudomonas syringae* van Hall.

The information obtained suggests that normal distribution curves give a correct indication of the effect of temperature on infection. From these curves the optimum temperatures for stem and leaf infection are 65°F. (18.2°C.) and 68.5°F. (20.1°C.) respectively.

Introduction

Blast of stone fruit caused by the bacterium *Pseudomonas syringae* van Hall is more serious in certain stone-fruit-growing districts in New Zealand than in others, and within the districts infection varies considerably between seasons and from year to year. Since methods of controlling this disease at present depend more on prevention of infection than on cure, it is important to know the conditions most suitable for development and spread of the causal organism. It is likely that spray schedules could be greatly reduced if we knew accurately when infection was most likely to develop, and it would be particularly advantageous to nurserymen and orchardists if serious outbreaks could be forecast.

This paper reports tests to determine the effect of temperature on infection by *Ps. syringae*.

Materials and Methods

For each test, twenty seedling peach trees (*Prunus persica* Sieb. and Zucc.) approximately 18 in. high, growing vigorously in pots in a glasshouse, were used as host plants. Stems were pricked lightly with a sterile needle at 20 points approximately 15 mm. apart, and 4 or 5 leaves were each pricked in 4 or 6 places to give 20 leaf inoculation points per plant, i.e. 400 stem and 400 leaf inoculation points per trial. The plants were then sprayed with a broth culture of a pathogenic strain of *Ps. syringae*. This inoculum was prepared by growing the organism for 24 hr. at 27°C. in Difco Nutrient Broth plus 0.5% dextrose. The number of viable bacteria, determined by plate count, ranged from 2.01×10^9 to 7.62×10^9 per ml.

After spraying with inoculum, plants were immediately placed in an insulated cabinet maintained at one temperature in the range 35.5°F. to 82°F. ± 2°F. (1.9°C. to 27.7°C. ± 1.1°C.) Relative humidity was maintained at 100% by adding a cold-water mist automatically from an atomizer for 40 second every 15 minutes. The cabinet was illuminated by natural sky light but was shaded to prevent entry of direct sunlight. After 7 days, plants were removed from the cabinet and results recorded by measuring lengths of stem lesions and diameters of leaf lesions (Fig. 1). Lesions measuring 1 mm. or less were recorded as negative owing to difficulty in distinguishing between weakly positive lesions and natural plant reaction to needle wounds.

Fig. 1.—Peach stems and leaf showing typical lesions caused by *Ps. syringae* after 7 days at 60°F.
Photo: J. W. Endt.

Experiments were carried out over a period of 18 months as supplies of trees came to hand at the correct stage of growth, i.e. growing vigorously and approximately 18 in. high. During this time 480 trees were used, the 20 trees for each test being always selected from a larger number in order to have as much uniformity as possible in vigour of plants.

RESULTS

Table 1 shows the mean sizes of stem and leaf lesions measured in each trial at the various temperatures. Mean size in this instance consists of total length or diameter of all positive stem or leaf lesions

greater than 1 mm. divided by 400. It therefore does not represent mean size of positive lesions. Table 1 also shows lesion sizes that would be expected if these results were to conform to normal distribution.

TABLE 1. OBSERVED AND EXPECTED MEAN LESION SIZES AT TEMPERATURES RANGING FROM 35°F. TO 82°F.

Stem			Leaf		
°F.	Observed mean lesion size (mm); average of 400 lesions	Expected mean lesion size (mm)	°F.	Observed mean lesion size (mm); average of 400 lesions	Expected mean lesion size (mm)
35.5	0.02	0.29	35.5	0.21	0.87
42	0.37	0.88	42	0.51	2.17
45	2.01, 1.43	1.33	45	1.55, 1.80	3.11
48		1.91	49	4.63, 4.21	4.68
49	1.19, 2.14	2.11	49.5	4.69, 5.99, 7.10	4.90
49.5	2.32, 3.07, 3.51	2.22	52	4.98	6.05
50		2.34	55	6.51, 8.59	7.49
52	2.93	2.79	56	9.51	7.97
55	2.02, 1.61	3.47	57		8.44
56	4.28	3.70	58	6.27	8.90
58	2.65	4.26	60	14.27, 10.48, 8.46	9.74
60	5.17, 6.10, 4.40	4.42	64	10.33	11.02
64	5.25	4.76	66		11.40
66		4.76	71	9.31, 9.53	11.40
70		4.42	73		11.02
71	3.75, 3.89	4.26	77		9.74
72		4.09	79		8.90
74		3.70	80	9.27, 8.79, 9.66	8.44
75		3.47	81		7.97
78		2.79	82	6.92	7.49
80	1.64, 2.29, 2.57	2.34	85		6.05
81		2.11			
82	1.42	1.91			
Mean of normal distribution		65			68.5
Standard deviation		12.5			14.5

Figs. 2 and 3 show normal distribution curves adapted to the data obtained in these experiments, together with the actual mean sizes of lesions on stems and leaves respectively at the different temperatures.

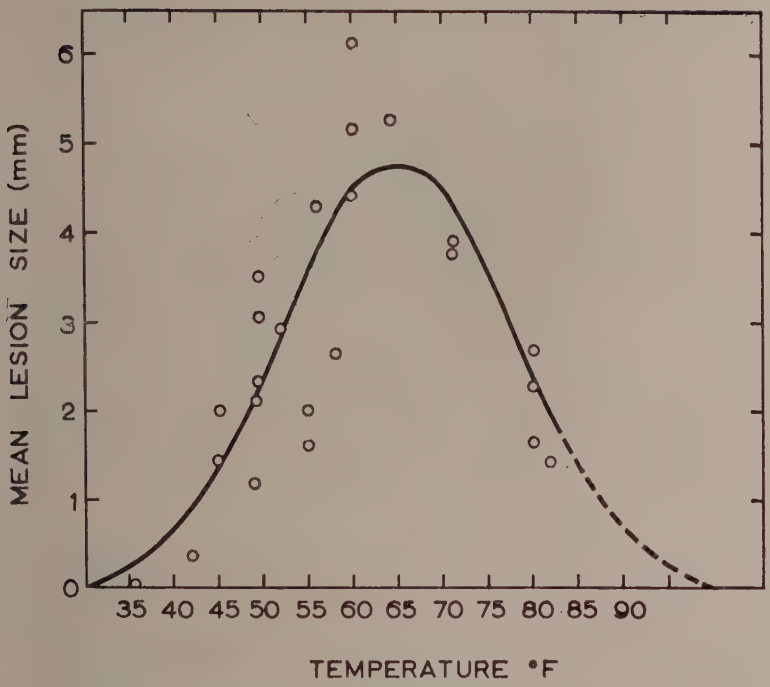

Fig. 2.—The effect of temperature on mean size of stem lesions. Normal distribution curve adapted to experimental data together with actual mean lesion-size for each experiment.

DISCUSSION

There was little leaf or stem infection at temperatures below 40°F. but it was found that the bacteria were not killed in plants at these temperatures. This was shown by the fact that when inoculated plants were held for 7 and 14 days at 35.5°F. followed by 7 days at 60°F., all became severely infected in the final 6 days. At approximately 80°F. stem infection was greatly retarded whereas leaf infection was little different from that recorded at 60–70°F. The observed mean lesion sizes (Table 1) indicate that optimum temperature for stem infection is within the range 58–71°F. (mean 64.5°F.) and for leaf infection 60–80°F. (mean 70°F.).

These findings are largely confirmed by observations of natural field infection. In Central Otago, the district most seriously affected with blast, average air temperatures during winter months are generally below 40°F. and very little blast infection is evident during this period. In spring average air temperatures rise steadily above 50°F. with daily maximum temperatures above 65°F. Infection is particularly serious at this time on stems and leaves. The average air temperatures during summer months are normally above 60°F. with maximum temperatures generally exceeding 80°F. At this time there appears to be no progression of stem cankers and few viable bacteria can be isolated from stem tissues. Leaf and fruit lesions however, develop steadily and contain

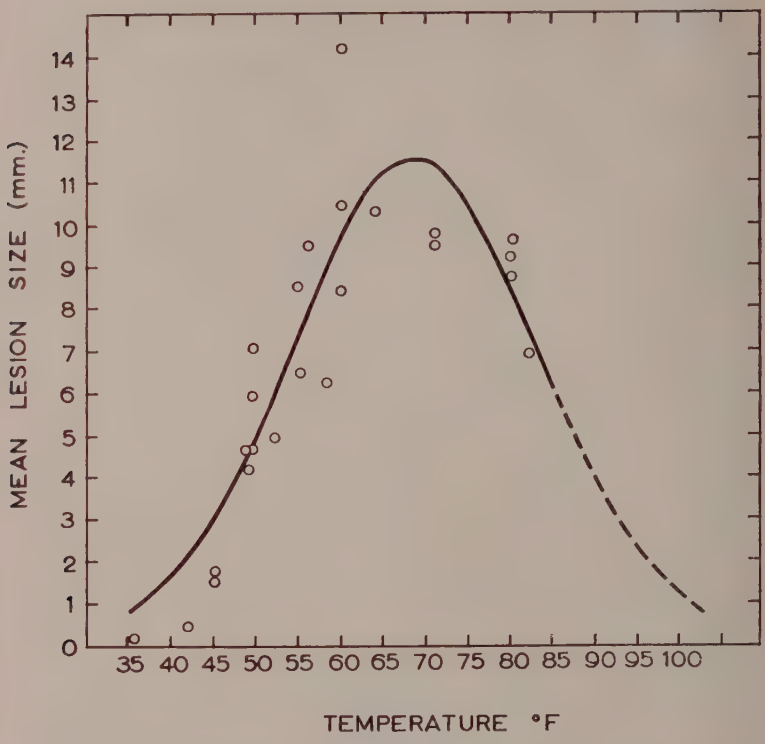

Fig. 3.—The effect of temperature on mean size of leaf lesions. Normal distribution curve adapted to experimental data together with mean lesion-size for each experiment.

masses of viable bacteria. In autumn months with average air temperatures falling below 60°F. infection again occurs in stems, particularly in young growth. There are of course other known factors which vary the amount of infection, in the field, such as humidity, age and species of host, and threshold number of viable bacteria, as well as other unknown factors.

With the information available at present, the normal distribution curves give a close approximation of the mean stem and leaf lesion size for most temperatures tested. There is insufficient evidence to prove that these curves do not give a correct indication of the effect of temperature on infection of peach trees by *Ps. syringae*. Variation in lesion size between trials at any one temperature was occasionally considerable but was not greater than that which sometimes occurred between plants in the same trial. Before these trials commenced, it was known that variation in infection was largely associated with growth rate of plants but although every effort was made to select plants having an even growth rate, some variation still occurred. One factor that was expected to cause variation between trials over a period of time was possible change in pathogenicity of the causal organism. However, it was not found difficult to maintain a high level of pathogenicity in the test

bacterium and it is felt that little variation was due to this factor. Subsequent experiments have shown that a 24–48 hour period of pre-treatment conditioning of plants at the particular temperature under test, would have done much to reduce the variation observed in these experiments.

If the normal distribution curves shown in Figs. 2 and 3 are accepted as being a correct representation of the effect of temperature on lesion size, the optimum temperatures for stem and leaf lesion development would be 65°F. and 68.5°F. (18.2°C. and 19.9°C.) respectively. These temperatures agree closely with those observed in the controlled temperature trials and with natural field infection. Further work will however be necessary before these curves can be used with confidence.

ACKNOWLEDGEMENT

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A COMPARISON OF GLASSHOUSE AND LABORATORY METHODS FOR TESTING FUNGICIDES AGAINST *Botrytis cinerea*

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(*Received for publication, 1 November 1956*)

Summary

A standard slide germination method using spores of *Botrytis cinerea* rated fungicides in almost the same order as that obtained in previously reported experiments on control of *B. cinerea* causing grey mould on glasshouse tomatoes. The major discrepancy between laboratory and glasshouse results was that the standard slide germination method gave copper fungicides a high rating, whereas in practice these materials were not effective in controlling grey mould on tomatoes. The reason for this anomaly was found to be that spores of *B. cinerea* can germinate on heavy deposits of copper in the absence of free water.

Introduction

The slide spraying technique for bioassay of fungicides, described by Horsfall (1945a), is mainly used for exploring new chemicals. Most of the work is done with a small group of test organisms, and the general fungicidal value of a material can be found by this method (Wellman and McCallan, 1943). Prediction from laboratory tests on standard fungi, of field performance of a fungicide against any particular disease is, however, uncertain, according to Keil *et al.* (1952), who compared dosage-response data from spores of *Stemphylium sarcinaeforme* (Cav.) Wilts. and *Sclerotinia fructicola* (Wint.) Rehm with data from field experiments on apple scab and bean anthracnose.

An attempt is being made in this laboratory to use the slide spraying technique for selecting from established fungicides those most suitable for field testing against particular pathogenic fungi. *Botrytis cinerea* Pers. was used as the test organism, since previous work (Brook, 1956) had established the relative performances of a range of fungicides against *B. cinerea* causing grey mould of glasshouse tomatoes. In glasshouse experiments, thiram, ziram, captan, dichlone, and ferbam had prevented grey mould on inoculated tomato seedlings, while maneb, salicylanilide, zineb, sulphur, and the coppers had been ineffective. Since dichlone caused leaf scorching, and ferbam left an objectionable residue, only thiram, ziram, and captan were tested on a glasshouse tomato crop. Of these, thiram gave the best performance.

The present paper describes results of spore germination tests on slides sprayed with the same fungicides used in glasshouse experiments.

Materials and Methods

As in the glasshouse, fungicides were in commercial preparations, as follows: captan (Flit-406); colloidal copper (Ialine Colloidal Copper); copper oxides (Perenox); copper oxychloride (Cuprox); dichlone (Phygon XL); ferbam (Fermspray); maneb (Manzate); salicylanilide (Shirlan A.G.); sulphur (Colsul 40); thiram (Thirospray); zineb (Dithane Z-78); ziram (Zirospray).

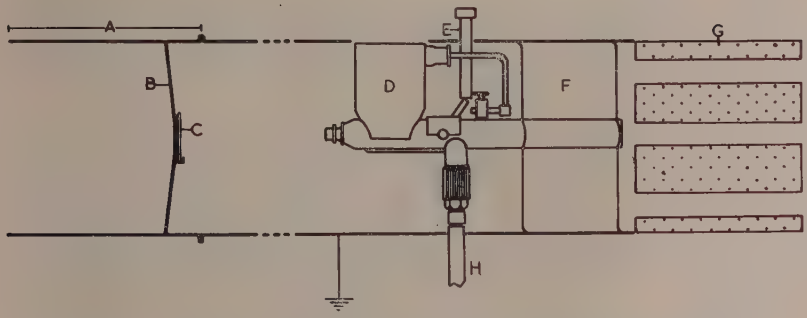

Fig. 1.—Horizontal spraying apparatus. A. Hinged portion of tube. B. Wire stay. C. Slide on carrier. D. Reservoir. E. Trigger mechanism. F. Supporting strap. G. Perforated porous brick. H. Air intake.

The apparatus is enclosed in a stainless steel tube of 4 in. diameter, with 30 in. between nozzle and slide.

The strain of *B. cinerea* used in laboratory assay was originally isolated from a diseased tomato and was the same as that used to inoculate the glasshouse plants.

Apparatus used for spraying nitrocellulose-coated glass slides (Fig. 1) was similar to that described by Horsfall (1945b). In the machine used here, humidity was provided from a perforated, saturated brick at the end of the tube behind the nozzle. The sprayer was an "Aerostyle Type M" air brush. The slide was held in a hinged portion of the tube and was swung into the spray stream for the required spraying time. Operated at an air pressure of 66 cm. mercury, the deposit was approximately 9×10^{-4} ml./cm.^{2} per second. A check on rate of deposit was made each day the apparatus was used.

Experiment 1

Fungicides were tested by the normal slide germination method. Spores from 7–10 day cultures on potato dextrose agar were washed in distilled water, centrifuged, and diluted to 50,000/ml. in 0.05% dextrose solution. Drops (0.05 ml.) of spore suspension were pipetted on to slides that had been freshly sprayed and air-dried. Spore germination was determined after 24 hours at 23°c. in humidity chambers. In control drops on unsprayed slides germination was 100%.

Details of dosage-response curves are given in Table 1. In the table, Group I includes those materials with comparatively steep slopes, and Group II those with flatter slopes. From tests with maneb and zineb only one point between 0% and 100% inhibition could be obtained by using X $\sqrt{2}$ dose increases, and slopes were not found for these fungicides.

Experiment 2

In Experiment 1, where spores were in water drops on the sprayed slides, LD 50 values for copper compounds were lower than that for thiram. This was in disagreement with results of the glasshouse experiments which showed coppers to be ineffective while thiram gave satisfactory control of grey mould. Doran (1923) found that dry spores

TABLE 1. DOSAGE-RESPONSE OF *B. cinerea* SPORES TO FUNGICIDES OBTAINED BY THE NORMAL SLIDE GERMINATION METHOD

Fungicide		m*	s^2†	LD 50 (μg./cm.^{2})	LD 95 (μg./cm.^{2})
Group I					
Thiram		1.326	0.031	0.212	0.406
Ziram		1.399	0.035	0.251	0.499
Captan		0.901	0.024	0.080	0.142
Dichlone		0.462	0.007	0.029	0.040
Maneb		2.070	—	1.170	< 1.430
Zineb		3.308	—	20.300	<28.400
Ferbam		1.893	0.014	0.782	1.210
Group II					
Copper oxychloride		0.440	0.296	0.028	0.204
Copper oxides		0.986	0.093	0.097	0.298
Colloidal copper		0.172	0.231	0.015	0.088
Salycilanilide		2.660	0.335	4.570	38.500
Sulphur		3.631	0.214	42.800	236.000
Approximate differences for significance:—					
Within Group I	5%	0.037	0.006		
	1%	0.048	0.008		
Within Group II	5%	0.117	0.048		
	1%	0.152	0.063		
Between Groups I & II	5%	0.087	0.035		
	1%	0.113	0.045		

* m = LD 50 on logarithmic scale.
† s = S.D. of line = reciprocal of slope.

of *Alternaria solani* (Ell. & Mart.) Sorauer could germinate on copper deposits although germination was prevented if spores were suspended in water on the deposits.

We found that dry spores of *B. cinerea* would germinate on dry slides in a saturated atmosphere, although germination was not 100%, and took longer than when spores were in dextrose solution. It could be expected that *B. cinerea* would behave in a similar way to *Alternaria solani* on copper desposits. To test this, a comparison of germination of dry spores of *B. cinerea* on slides sprayed with thiram and with copper oxychloride was made. In order that deposits should be of uniform distribution at all doses, spraying time was kept constant at 40 seconds and concentration of the spray mix was varied. Spores from petri-dish cultures were brushed on to the dried deposits and slides held at 23°c. in humidity chambers. After 30 hours, germination counts were made.

Since germination on control slides was only 42%, accurate dosage-response curves were not obtained. Percentage germination at different dosages is shown in Table 2. While complete inhibition occurred with 23.5 μg./cm.^{2} of thiram, germination on the copper oxychloride deposits, even at 211 μg./cm.^{2}, was not greatly different from that on unsprayed slides.

The effect of still higher dosages of copper oxychloride could not be examined since the spray apparatus would not handle the heavy fungicide concentrations needed.

TABLE 2. PERCENTAGE GERMINATION OF *B. cinerea* SPORES ON DRY DEPOSITS OF THIRAM AND OF COPPER OXYCHLORIDE

	Dose (μg./cm.²)					
	Nil	2.61	7.83	23.5	70.5	211
Thiram	42%	54%	7%	0	0	—
Copper oxychloride	42%	—	53%	31%	30%	48%

Note: 0 = no germination
— = fungicide not tested at the dose.

Experiment 3

A lower LD 50 was obtained for captan than for thiram in Experiment 1. In preliminary glasshouse experiments captan appeared to be as good as thiram. However, over a whole season on a glasshouse crop thiram performed more satisfactorily than captan.

As a result of the finding that spores of *B. cinerea* could germinate without free water on copper deposits, the possibility that captan might not be as effective as thiram against dry spores was examined. It was found, however, that when dry spores were brushed on to dry sprayed slides captan at a dose of 1.29 μg./cm.² caused complete inhibition of germination compared with 11.6 μg./cm.² of thiram required to give the same result.

Another possibility is that deposits of captan are less stable than those of thiram. If this were so, by the end of the fortnightly period between two spray applications to a glasshouse crop the captan residue might be relatively ineffective while thiram continued to give protection. On a glasshouse crop, where wind and rain are absent, weathering is restricted to effects of light and exposure to the atmosphere. To test these effects on breakdown of the two chemicals, triplicate series of slides were sprayed, one series being inoculated with drops of spore suspension as soon as dry, the second after 3 days, and the third after 9 days. The second and third series were exposed to normal daylight on a bench in a well-lit room until due for inoculating. LD 50 values are given in Table 3.

TABLE 3. DOSAGE-RESPONSE OF *B. cinerea* SPORES TO AGED DEPOSITS OF THIRAM AND CAPTAN

Fungicide	Age of deposit	m (log.LD 50)	LD 50 (μg./cm.²)
Thiram	2 hrs.	1.413	0.259
„	3 days	1.379	0.239
„	9 days	1.416	0.261
	Sig. diff. 5%	0.015	
	1%	0.020	
Captan	2 hrs.	0.849	0.071
„	3 days	0.711	0.051
„	9 days	0.847	0.070
	Sig. diff. 5%	0.030	
	1%	0.039	

The low LD 50 values for both captan and thiram in the 3-day series have still to be explained. However, since the LD 50 values for both materials on the ninth day showed no increase over those on the first day, it appears that exposure to daylight and air is unlikely to be responsible for any major breakdown of either fungicide over the periods between spray applications in the glasshouse.

Discussion

There is, in general, fair agreement between results of laboratory assay by the standard slide germination method and results of earlier glasshouse work, (Brook, 1956). Maneb, zineb, salicylanilide, and sulphur, which were ineffective in controlling grey mould, were found to have relatively high LD 50 values in the laboratory. Thiram, ziram, dichlone, and captan, which were all effective in preliminary experiments on disease control in the glasshouse, had comparatively low LD 50 values. Dichlone, however, was found in the glasshouse to be phytotoxic. Ferbam, which took only an intermediate place in the laboratory order, performed well in the preliminary glasshouse experiments but was not used for a season on a crop, so that a complete evaluation of this material in the glasshouse is not available.

The outstanding discrepancy between results obtained by the normal slide germination method and those from glasshouse experiments was the difference in rating of copper compounds. In the glasshouse, copper fungicides were of little or no value in controlling grey mould on tomatoes, but in the laboratory when the normal technique was used LD 50 values for three copper compounds were lower than those of nearly all other materials. This discrepancy was explained when it was found that spores of *B. cinerea* were able to germinate without free water on deposits of copper which were many times heavier than those required for complete inhibition when the spores were suspended in water. Several sulphur-containing organic fungicides, on the other hand, are known to have volatile, toxic, decomposition products, (Weed *et al.*, 1953). Thiram and captan probably owe their effectiveness in preventing germination of dry spores of *B. cinerea* to toxic gases developed from their deposits.

It is clear, from comparison of laboratory with glasshouse results, that, even when the organism causing the disease is used in the laboratory, spore toxicity tests cannot be depended upon to predict accurately the relative merits for disease control of the most promising group of fungicides. Artificial weathering tests assist selection of suitable compounds for outdoor crops, (Keil *et al.*, 1952), but in the present work, which concerned a glasshouse crop, a simple aging of spray deposits threw no light on why thiram and not captan should give the most satisfactory control of grey mould. Unfortunately, laboratory tests cannot predict the importance of the plant factor in disease control.

It does seem, however, that for a particular disease, laboratory assay on the causal fungus can result in a useful selection of those materials worth field testing from the wide range of established fungicides now available.

Acknowledgement

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SEEDLING STUDIES IN FESCUE-TUSSOCK GRASSLAND

II. PERFORMANCE IN FOUR SITES

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Summary

At Molesworth Station, Marlborough, New Zealand, following preliminary trials with counted grass seeds, small plot experiments have been continued in four contrasting sites. All seed sown in drills germinated well but in only one site did the seedlings survive without the protection of litter, and here after two seasons plants are well established; elsewhere seedlings could not withstand the action of frost in winter followed by heavy spring rains and desiccating summer winds. In surface-sown plots germination varied from fair to nil, but in none did any plants survive. Where seedlings had failed to establish in earlier trials it was found that heavy litter laid on immediately after sowing definitely aided establishment of seeds sown in drills in November; on seeds similarly sown in autumn, and on those surface-sown in spring or autumn, litter produced no long-term beneficial result. Effects due to aspect and the influence of litter on other sites are now being studied.

Introduction

This paper records results of work in fescue-tussock grassland at Molesworth Station, Marlborough, further to that already described (Simpson and Moore, 1955). The first set of experiments sampled only one inter-tussock habitat, where a small annual *Gnaphalium* (closely related to *G. japonicum*) gives a characteristic appearance in summer. *Festuca novae-zelandiae* tussock grassland at Molesworth, however, is a complex community and several different types of inter-tussock space can be recognized, each with its own particular assemblage of plants. On the same ridge top as before, four different sites within a few hundred yards were chosen for the experiments described here.

Most of the common species of the inter-tussock spaces of the ridge-top appear in Table 1, which records the species present in the experimental plots in autumn, a year after the last sowing of seeds. Though this is not an ideal sampling of the sites, it does in fact give a fair picture of the floristic similarities and contrasts between them; a list made in the same way a year earlier is almost identical. In early summer more annuals would probably be recorded, and the total number of species for the ridge top is greater than that recorded for all the plots.

Rainfall records for the period are summarized in Table 2. The season 1953–54 was good for growth, and conditions in general were at an optimum for the district. Soil temperatures at a depth of two inches were measured in July and October at Site 1 under bare ground and under litter. Figures for diurnal temperature ranges (max.-min.) for soil at one inch depth on sunny days in October on the same ridge have been published, as have also notes on ice forms under bare soil in July 1954 at one of the sites (Gradwell, 1955, Tables 3 and 5).

TABLE 1. SPECIES PRESENT IN EXPERIMENTAL PLOTS IN APRIL 1955
Number of plots out of four at each site in which each species was present
A = adult plants, S = seedlings; * non-indigenous species.

	Site 1		Site 3		Site 4		Site 2	
	A	S	A	S	A	S	A	S
**Rumex acetosella*	4		4		4		4	
**Holcus lanatus*	4	1	3	4	4	1	4	
Epilobium hectori	4		4		4	2	3	
**Crepis capillaris*	4	1	4	4	4	2	3	4
Poa lindsayi	3		4		3		4	
Geranium sessiliflorum	1		1		2		2	
Acaena sanguisorbae var. *pilosa*	1		2	1	2			
Wahlenbergia albomarginata	3		2				1	
Tortella calycina (green moss)	2		3					
Agropyron scabrum		3		3	2	1		1
**Cerastium* sp.	3	3	1	1				3
Gnaphalium japonicum (?)	3		4		1			
**Hypochaeris radicata*			2		3			
**Gilia squarrosa*			4					
**Agrostis tenuis*			2					
**Pinus murrayana*				2				
Gnaphalium luteo-album			4		3	1		
Tillaea multicaulis					4			
Festuca novae-zelandiae					2	2		
Epilobium rostratum var. *pubens*							4	
**Erodium cicutarium*							4	4
**Verbascum virgatum*							4	
**Arenaria serpyllifolia*	1						4	
**Veronica verna*							3	
Tortula princeps (brown moss)							3	
**Poa pratensis*							2	
Raoulia australis	1						2	
**Rosa eglanteria*	2						3	3
Cladonia sp. (lichen)	1						1	
**Myosotis versicolor*	2						1	1
Total number of species	19		18		13		24	

Additional species recorded in only one plot are:
in site 1, *Raoulia lutescens*, **Gnaphalium collinum*;
in site 3, **Dactylis glomerata*; in site 2, **Taraxacum officinale*, **Cirsium lanceolatum*, **Verbascum thapsus*, **Bromus mollis?*
Worm casts were recorded in all plots in site 2, and in half the plots in other sites.

METHODS

At each site plots were laid down as follows: (a) seed sown in drills in November 1953; (b) seed sown in drills in March 1954; (c) seed scattered in quantity on the soil surface in November 1953; (d) seed scattered in quantity on the soil surface in March 1954. The species used in each plot were *Agropyron intermedium*, *Bromus inermis*, and

TABLE 2. RAINFALL (INCHES) AT MOLESWORTH 1953-55
(Number of rain days in brackets)

	J.	F.	M.	A.	M.	J.	J.	A.	S.	O.	N.	D.
1953									1.22	1.96	2.38	4.69
									(8)	(8)	(11)	(9)
1954	0.96	0.68	4.94	2.16	3.40	3.35	2.74	2.89	0.56	0.39	1.56	3.74
	(4)	(3)	(10)	(8)	(10)	(6)	(7)	(8)	(4)	(4)	(8)	(9)
1955	1.74	4.94	0.98	1.07	2.35	3.08	3.71	3.42				
	(7)	(11)	(4)	(6)	(8)	(10)	(8)	(13)				

Bromus maritimus. In addition *Agropyron scabrum*, local Molesworth strain, was used in Site 1. Drills were cultivated to a depth of four to six inches and seed was then sown two inches deep. In all plots, seed was sown at a heavy rate. At Site 1 (that described in 1955) four comparable plots sown in the same way were covered at the time of sowing with litter in the form of rather tight tufts of dead foliage from plants of *Festuca novae-zelandiae*. Litter was applied fairly heavily and had a pronounced effect on light, humidity, and temperature. Each plot was covered by a frame measuring 48 × 30 × 6 in. with slightly sloping sides, made of heavy cyclone netting. The frames afforded some protection from wind and light.

DESCRIPTION OF SITES AND RESULTS

Site 1

This is the site described in detail in the former paper. The tussocks occur in small clumps or singly with centres ranging from three to eight feet apart. Many are gradually disintegrating. The soil is dark in colour and comparatively free from stones. There are some worms present and numerous fibrous roots of *Rumex acetosella*. Drainage is not good and water collects in the hollows from melting snow and early spring rains.

Seed Sown in Drills in November 1953

The germination of all three species was good. By March 1954 growth of *Bromus maritimus* and *Agropyron intermedium* reached up to 6 in. in height, this being the maximum growth made in any of the four sites. *Bromus inermis* was smaller here.

In May this plot had the strongest development of needle ice; some fairly large blocks of soil were lifted up on top of needles. Here, where growth of the seedlings had been sufficient to afford some protection to the soil, more ice was formed in the inter-row spaces. *B. maritimus* had grown particularly well. By October, however, the two good rows of this species were completely disrupted and *B. inermis* and *A. intermedium* were left on the more or less compact soil blocks; one corner of *A. intermedium* had been completely lifted out. A large

number of *B. inermis* plants, especially at the ends of the rows, had most of their roots exposed. After two fairly wet months, some plants were still growing in January 1955 on top of the soil hummocks, some at least two inches above the general surface of the soil. Many had died as the soil fell away from the roots and had the summer been drier the loss would have been much worse. All *B. maritimus* plants were dead in January. By March it was evident that all other plants were gradually dying as the soil dried away from the roots. Fig. 1. After a further winter no plants survived and the plot was abandoned in the following spring.

Fig. 1.—Site 1. Young plants left on soil blocks several inches above the general level of the plot. Sown in drills November 1953; photo. March 1955. These plants died when the hummocks were broken up during the following winter. *A. intermedium* to left of peg, *B. inermis* to right, two rows of each. On extreme right a few plants of *A. scabrum*. *B. maritimus* in two rows on extreme left had already died.

Photo: W. R. Boyce.

Seed Sown in Drills in March 1954

Germination was good; by May plants were reasonably well grown. There was some needle ice between the rows, but at that time not immediately affecting the plants. By October *B. maritimus* was completely lifted out and all plants were dead. *A. intermedium* and *B. inermis* survived for a short period on the soil hummocks but gradually died off over the summer and the plot was abandoned.

Surface-sown in November 1953

B. maritimus germinated very well and some seedlings were 4 in. high in March. *A. intermedium* and *B. inermis* started to germinate after heavy rain in early March. In May, needle ice was $1\frac{1}{2}$ in. deep over the plot, but there had not been sufficient thaw to cause much damage. By October, however, all the seedlings had been heaved out and the plot was abandoned.

Surface-sown in March 1954

Germination of all species here was very poor; the seedlings died over the winter and the plot was abandoned in October.

Effect of Litter on Temperature

Soil temperatures at a depth of 2 in. taken in July between 11 a.m. and 1 p.m. gave a range of 33.3° to 34.6° in four litter-covered plots compared with 32.3° to 33.9° in four plots without litter (pers. comm., M. W. Gradwell). On 2 October 1954 readings at 2 in. depth in soil taken at intervals from 6 a.m. to 9 p.m. gave a range from 38° to 66° under litter compared with 36° to 72° in plots without litter. (cf. Gradwell 1955 Table 5 at 1 in. depth). From 10 a.m. to 9 p.m. temperatures were generally higher in plots without litter, the greatest recorded difference being at 3.30 p.m. All control plots had lower temperatures than the litter-covered ones at 6 a.m.

Seed Sown in Drills in November 1953, with Litter

Germination was exceptionally good, and by March some of the seedlings were topping the frames which are six inches high. Some *Rumex acetosella* was growing through the litter. In May, soil was moist and friable and there was no development of needle ice under the litter. After the winter all species continued to make good growth and roots were firmly established. The deep litter had not induced etiolation or

Fig. 2.—Site 1. Seedlings established through litter. Sown in drills Nov. 1953. Photographed August 1955. From left: *A. scabrum*, *B. inermis*, *A. intermedium*, one row of each; a row of *B. maritimus* on extreme right had already died. Note litter around plants. Peg 7 in. high.

Photo: M. J. A. Simpson.

weakening of growth. At the edges of the litter the soil was lifted and pulverized by frost needles. In January 1955, *B. maritimus* had deteriorated but *A. intermedium* was about 15 in. high and *B. inermis* had tillered out very well. These two were better than in any of the other plots. At this time the soil beneath the litter was damp and cool and little other growth had appeared. At later dates plants here were all tall, strong, and well developed. Fig. 2.

Seed Sown in Drills in March 1954, with Litter

Germination of all species was poor and by October only one or two plants were showing in each plot. The soil beneath the litter was moist and friable and had not been disturbed by frost. In January 1955 there were no seedlings in the plot and no seed could be found. There was much earthworm activity beneath the litter.

Surface-sown in November 1953, with Litter

B. maritimus germinated very well and nearly every seed of *A. intermedium* appeared to have germinated. Growth was very good and a thick mat of foliage had penetrated the litter. The germination of *B. inermis* was also good and plants showed through the litter. In this plot a number of *Poa lindsayi*, *Holcus lanatus*, and *Rumex acetosella* were showing and also some *Epilobium hectori*. In May 1954, the growth appeared much better than in the plot with seed sown in drills, but during the winter a large number of plants died. By October, *B. inermis* was the only species with roots penetrating the soil, and plants seemed reasonably established, but in January 1955 there were only a very few left alive together with some *A. intermedium* plants which possibly came from a spring germination. In March, just a few thin shoots were showing through the litter and by August there was nothing left and the plot was abandoned.

Surface-sown in March 1954, with Litter

Here in contrast to the plot sown in drills in March, the germination was good, and, by May, seedlings of all species were growing nearly two inches through the litter. The ground was not frozen beneath the litter but there was a good deal of needle ice at the exposed edges. Again, however, the seedlings did not succeed in establishing sufficiently and the plot had to be abandoned in the following spring.

Agropyron scabrum. Seed of *A. scabrum* was sown in every plot in Site 1. Germination was very good except in the surface-sown plots without litter and in the litter-covered plot drill-sown in March 1954. In most plots not many plants survived the first winter and these later died, a few after producing seed heads. In contrast, in the litter-covered plot drill-sown in November 1953 a full row has established (Fig. 2).

Site 2

This is a fairly large open space, about twelve yards wide, parallel and close to the crest of the ridge, with a slope of less than 5° to the S.S.W. There are a few debilitated tussocks clumped here and there along the slope, but the most numerous larger plants are *Holcus lanatus*, *Verbascum virgatum*, and *V. thapsus*, with occasional mats of *Raoulia australis* and *R. lutescens*. Generally, the cover is very sparse and a

few hundred yards to the east the slope passes into a very depleted ridge with few plants except *R. lutescens* (scabweed). The soil is dark in colour and stony throughout, with a surface layer of smaller stones. Drainage is good, owing partly to the slope and in part to the open texture of the soil surface. The important plants are *Rumex acetosella, Epilobium rostratum* var. *pubens, E. hectori, Crepis capillaris,* and *Poa lindsayi.* Fig. 3.

Fig. 3.—General view of experimental ridge with Site 2 in foreground. March 1955.

Photo: W. R. Boyce.

Seed Sown in Drills in November 1953

By mid March 1954, seedlings were well established and about 6 in. high. Some leaves had been burnt at the tips with recent frost. On 26 May there had been no development of needle ice and further growth was noted. After the winter, the soil between the rows showed signs of frost action, and local soil movement had twisted rows in the upper part of the plot, but few plants had died. *B. maritimus* was frosted back to ground level during the winter but new growth three inches tall was showing in October. The other two species also had much new growth at this stage. At the ends of the rows a few plants had roots badly exposed and some were lying dead on the soil surface. By January 1955, *A. intermedium* and *B. inermis* had begun to tiller out and later observations indicated that plants in this plot were comfortably established (Fig. 4).

Seed Sown in Drills in March 1954

Germination of all three species was again good with growth up to three inches by May. *A. intermedium* germinated better from this sowing. Again in October there were signs of frost action between the rows, but no plants had been lost because of this and few roots had been exposed. *B. maritimus* plants here were all dead and the soil where

Fig. 4.—Site 2. Sown in drills in November 1953; Photographed August 1955. From left *B. maritimus*, *A. intermedium*, *B. inermis*, two rows of each.

Photo: M. J. A. Simpson.

these two rows were had been moved down the slope. More seedlings appeared at this time. *A. intermedium* and *B. inermis* are now reasonably well established, although both less advanced than those from the November sowing.

Surface-sown in November 1953

There had been little germination here by 12 March 1954, but after heavy rain on 16 March a good percentage of each species germinated. By May, the seedlings of *B. maritimus* were as advanced as those in the drill-sown plot, and *B. inermis* were also quite well grown. *A. intermedium* seed remained in the soil surface with a very poor germination. More seedlings of each species appeared in the spring and together with the older plants made reasonable growth, but none were able to get their roots sufficiently established in the ground, and all slowly and gradually died out over the summer; the plot was discarded in March 1955.

Surface-sown in March 1954

B. maritimus did not germinate. A few *A. intermedium* and *B. inermis* seedlings appeared before the winter. Further germination the following spring was poor and the plot was abandoned in January 1955, Fig. 5.

Site 3

This is on a slope of about 13° facing S.E. where there is some small terrace formation. Tussocks are approximately three feet apart

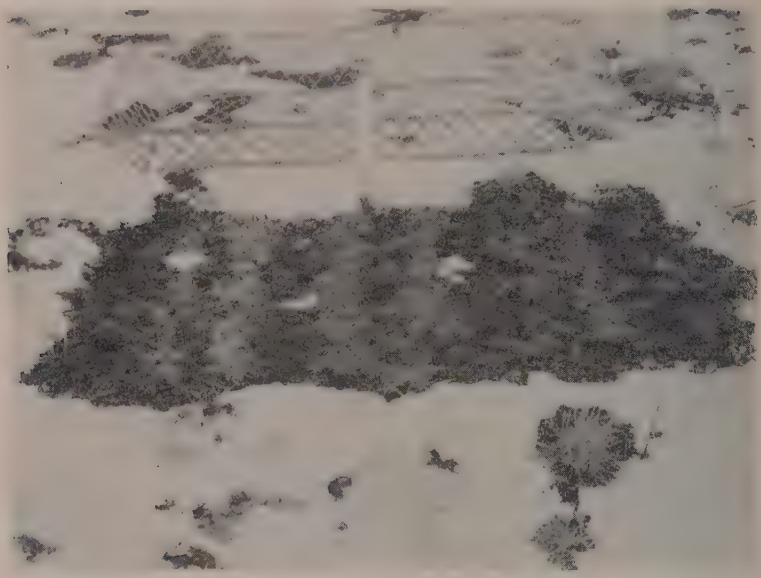

Fig. 5.—Site 2. Ungerminated seed on soil surface July 1954. Grass plants in right foreground are *Holcus lanatus*.

Photo: H. R. Penhale.

Fig. 6.—General view of Site 3. March 1956.

Photo: Nat. Publicity Studio.

with larger bare patches where the small terraces are forming. A shelter belt of *Pinus murrayana* to the north partly shades the site. The soil is dark brown and there were numerous worms present. A few medium-sized stones 6–8 in. in diameter are scattered on the surface. Drainage is not good and the soil in the terraces remains moist most of the year. Important plants are *Gnaphalium luteo-album, Gilia squarrosa, Gnaphalium japonicum* (?), *Poa lindsayi, Crepis capillaris, Epilobium hectori, Rumex acetosella,* and *Hypochaeris radicata.* Fig. 6.

Seed Sown in Drills in November 1953

The germination was patchy and that of *B. maritimus* very poor, but some plants of all three species were sturdy and well developed by March 1954. All the rows in the plot and in particular those of *A. intermedium* had moved downhill over the summer, showing that some soil movement had taken place. As early as May, needle ice was strongly developed and plants were being heaved out. After the winter all plants of *B. maritimus* had been lifted out by frost action. *A. intermedium* plants were still alive but lifted high in small hummocks. Only a few plants of *B. inermis* were alive and they too were uplifted on soil blocks. A number of dead plants were lying on the surface of the soil, and plants at the edges of the soil clumps were also dead. The ground, apart from the detached soil crumbs, had not dried out.

By January 1955 there were no remaining plants of *B. maritimus.* A few *A. intermedium* were still alive but their roots were becoming more and more exposed. One small clump of *B. inermis* had settled back into the ground and the remainder had died as the soil was blown away from the roots. In the more open patches a good cover of moss developed. The soil was very compacted but became more friable as it dried out over February and March. Plants did not succeed in settling back into the ground and frost action during the second winter was sufficient to remove all the seedlings and the plot was finally abandoned in September.

Seed Sown in Drills in March 1954

A. intermedium and *B. inermis* showed a good germination by May. *B. maritimus* did not germinate at all and by October the seeds had been thrown out on to the soil surface. Nearby, some fairly large plants of *Holcus lanatus* had also been thrown out of the ground. At this later date *A. intermedium* plants from two well defined rows were all lifted out and seemed unlikely to survive, and *B. inermis* were all dead. The surface over the whole plot was very broken and some general downhill movement of the soil could be noted.

By January 1955, only a few *A. intermedium* plants were alive and ungerminated seeds of all species were lying on the ground. One of the plants of *Holcus lanatus* was dead on a hummock 3 in. high. By March there were no sown seedlings surviving and the plot was abandoned. Many *Epilobium* seedlings were noted on this visit.

Surface-sown in November 1953

A few seeds had germinated in March 1954, the seedlings being about one inch high and still at the first-leaf stage, and the roots had barely managed to penetrate the ground.

After the winter there were no living seedlings left and no further germination, and the plot was abandoned.

Surface-sown in March 1954

Germination by May was fair but the majority of seeds were frozen to the soil surface. Of the four plots in this site, needle ice was deepest and most severe here and extended all over the plot at this time. A large number of *A. intermedium* seeds remained ungerminated but there was one good patch with roots reasonably well developed. A small patch of *B. inermis* survived the winter and settled back into the soil. *B. maritimus* here did not germinate at all. By January 1955 all the seedlings had died and the plot was abandoned.

Site 4

Here the slope is less than 3° to the S.S.E. There is a wide space without any tussocks and those around the periphery are spaced far apart. The soil is yellow brown with very few worms. In the summer the surface becomes very compacted and dry and polygonal cracks appear. Cultivation of the soil here increased the amount of needle ice formed in the winter.

Important plants are *Tillaea multicaulis, Rumex acetosella, Holcus lanatus,* and *Poa lindsayi.* Fig. 7.

Fig. 7.—Portion of Site 4. This plot was sown in drills in November 1953 and photographed in March 1955 when the *A. intermedium* in the foreground and a few *B. inermis* around the peg were all that remained. These failed to survive the following winter.

Photo: M. J. A. Simpson.

Seed Sown in Drills in November 1953

Germination of *B. maritimus* was good. In the first season, plants reached a height of 4 in., which is as good as would be expected in

nursery rows at Molesworth. The germination of *B. inermis* was poor and only a few plants were established by the end of summer. Germination of *A. intermedium* was fair but less than that in other plots. By March 1954, displacement of the rows was quite noticeable. In May there was strong development of needle ice and in the rows plants were already being heaved out. All plants of *B. maritimus* were lifted out of the ground by October. Six plants of *A. intermedium* remained alive on top of soil crumbs, and *B. inermis* plants, although still in defined rows, were well lifted up and at least half of them had died. Ungerminated seeds had been thrown out of the ground. Even *Rumex* plants in this plot had been lifted up and exposed, and in May the tops were withered back. Gradually the soil surface broke up and by summer it was quite hard and very dry. In January 1955, *B. inermis* still formed a well-defined row with many good plants, but a number had died. Plants were close together and the roots no doubt helped to keep the soil compacted. The small clump of *A. intermedium* was still living and a few *B. maritimus,* the latter, it is suspected, from a spring germination. By March there was a bare representation of each species and these died during the following winter as a result of further frost activity.

Seed Sown in Drills in March 1954

Germination of three species was very good, some of the seedlings in May measuring as much as three inches. At that date needle ice one inch deep was present in all inter-row spaces. All plants of *B. maritimus* had been lifted out completely and were dead. After the winter the roots of *B. inermis* plants were much exposed and those of *A. intermedium* had been completely lifted out of the soil and plants were lying dead on the soil surface. Ungerminated seeds had again been thrown out of the ground. Some *Rumex* and *Holcus* plants were lifted on hummocks. By January 1955, practically all the plants in the plot were dead. Small hummocks with included plants were scattered about the plot and these plants too were nearly all dead. The plot was abandoned in March when no plants of any of the three species survived. Roots had all been bared by frost heave and then dried by summer winds. The soil surface by the end of the summer was again very hard and bare and in places cracked. Seed that had been thrown out of the ground was being blown about and dead plants included in small detached soil hummocks were scattered as the soil dried around them.

There were numerous worm casts, and seedlings of *Tillaea multicaulis* were appearing for the first time this season. It is not known whether this species is always an annual or behaves as such only in particular situations. Inside the disturbed plots the plants were much fewer than in a comparable area outside.

Surface-sown in November 1953

Here germination was very poor and only a very few seedlings had emerged by March 1954. In May there was no development of needle ice in this plot. There were no seedlings alive by October and some plants of *Festuca novae-zelandiae* and *Agropyron scabrum* had been lifted out over the winter, but where there was sufficient cover of *Rumex* etc., the soil was already settling back into place. Many ungerminated seeds remained on the soil surface with no further emergence and the plot was abandoned in October.

Surface-sown in March 1954

Germination was again poor and in May there was no needle ice. In the following spring a little *A. intermedium* and *B. inermis* germinated but did not survive long, although some of the seedlings had worked down amongst the *Rumex* roots, rabbit droppings, etc., which were present in the plot. The plot was discarded in the summer when the few seedlings present failed to survive the summer wind and drought.

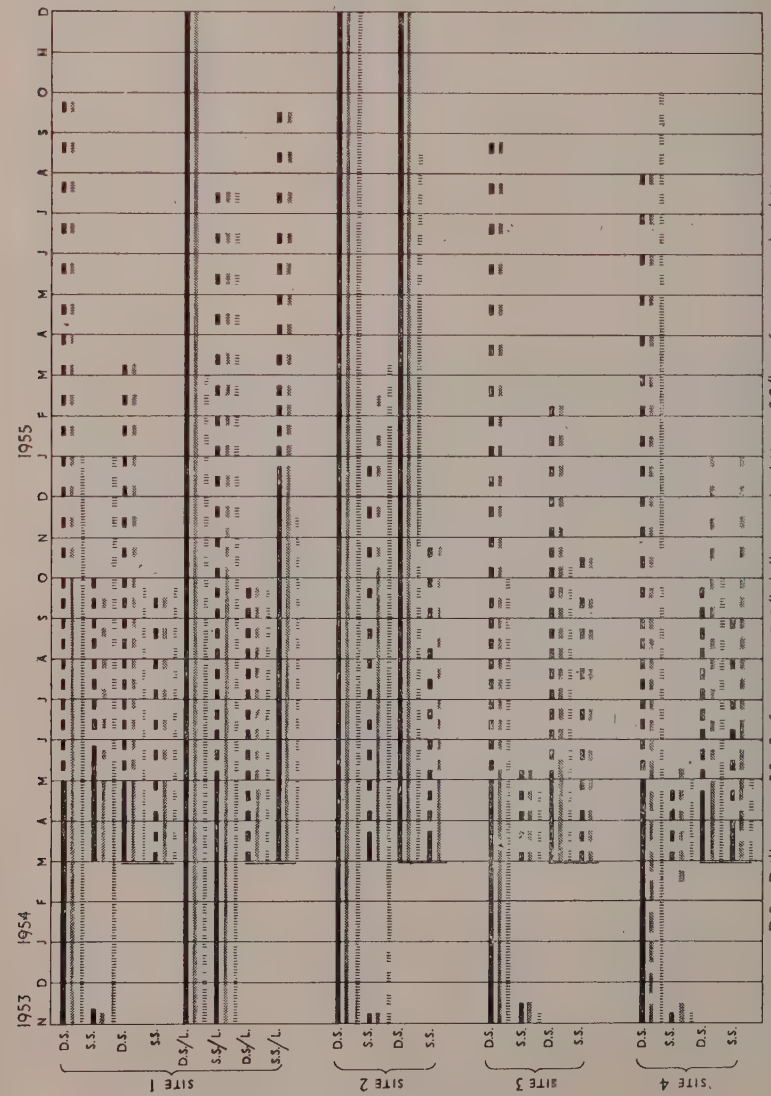
1953
1954
1955
N D J F M A M J J A S O N D J F M A M J J A S O N D
D.S.
S.S.
D.S/L.
SITE 1
SITE 2
SITE 3
SITE 4
D.S. = Drill sown. S.S = Surface sown, D.S/L drill sown plus litter. S.S/L=surface sown plus litter.
Broken lines show period over which plants died.
=Agropyron intermedium.
=Bromus inermis
=Bromus maritimus

Fig. 8.—Summary of performance of three species in four sites.

DISCUSSION

Figure 8 summarizes results for all plots, but does not include *Agropyron scabrum,* sown only in Site 1.

Germination of seed sown in drills was good in all four sites, but from the surface sowings the percentage was low in most plots and seedlings that survived at all were mostly in hollows, in the shade of plants where dew formed, or at the bottom of an accumulation of seeds. Heavy rain on 20 December 1953 did not promote general germination of surface-sown seed, but very active germination in nearly all rows was reported in mid April 1954. Seed lying on the ground was watched for signs of damage by mice or insects but none was lost in this way and most was eventually blown away when it dried during the summer. In none of the surface-sown plots were the seedlings that did grow able to penetrate the ground sufficiently to become permanently established. As in previous trials, the *Bromus* species germinated better from spring sowing and *Agropyron* species were better when sown in autumn.

Rows sown in November were moved downhill by the following March both in Site 3, where there is terracette formation on a slope and the ground remains wet most of the year, and in Site 4 where the slope is not great. In Site 2, rows were displaced during winter, particularly near large surface stones, and in Site 1 rows were also disrupted as a result of frost action. Some little damage to the surface soil is caused when heavy spring rains come before the thaw is complete and blocks of soil with included ice needles two inches long have been seen being moved by rainwater under such circumstances.

Where the soil had been cultivated, and in particular where there was little protection because of low germination of autumn-sown seed, the development of needle ice was more severe than in undisturbed soil. Thickly sown ungerminated seed lying on the surface decreased the amount of needle ice nearby.

Frost was not equally effective in all types of inter-tussock space. Only in Site 2, where aggregation of small stones and slight slope were sufficient to reduce the formation of ice needles, have unprotected *B. inermis* and *A. intermedium* established successfully; *B. maritimus* established here too, but under Molesworth conditions this species tends to behave as a semi-annual. A protective cover of litter allowed drill-sown seedlings to establish and survive in Site 1 where unprotected controls succumbed and where, in the previous season, ninety to one hundred per cent of counted seedlings had been killed during the first winter by frost action. Here *Agropyron scabrum* benefited from the litter in the same way as did the non-indigenous grasses.

These experiments have confirmed that sites recognized as distinct because of contrasts in their plant cover do in fact respond differently to sowing. Trials in hand include greater slopes of several aspects, and the effects of litter are being further investigated.

ACKNOWLEDGEMENTS

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EFFECT OF A PERMANENT GRASS COVER ON APPLE-TREE YIELD IN THE FIRST YEARS AFTER GRASSING

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(*Received for publication, 17 September 1956*)

Summary

An experiment has been initiated in the Nelson district to assess effects of a grass and clover sward on apple-tree growth and production.

In the first six years a reduction in yield occurred in trees under grass. Addition of extra nitrogenous fertilizer increased the yield of trees under both grass and clean cultivation. The yield of trees under grass and with this extra nitrogen was not as high as trees under clean cultivation and without extra nitrogen. Omission of phoshpate did not reduce yield under either grass or clean cultivation.

Introduction

The usual method of orchard-soil management in New Zealand is clean cultivation for most of the year. In the autumn, a cover crop may be sown or volunteer weed growth allowed to come away. Soil is ploughed or disced in the spring and then kept cultivated till autumn. This method has the advantages of retaining plant cover on the soil during times of heaviest rainfall and of keeping the soil clean during summer months when plant growth could compete with fruit trees for moisture.

Particularly in the Nelson district, however, where slopes are steep, topsoils shallow, and rain showers sometimes heavy, there are objections to this method of soil management, particularly from the point of view of soil conservation. Any method of management that would prevent soil erosion must have long-term advantages. Among methods that have been suggested for avoiding erosion is the sowing of a permanent grass sward in the orchard.

Permanent grass with grazing sheep is a traditional method of orchard management. However, our bush trees and modern spray programmes now preclude the use of sheep. In England, where interest in grassing-down orchards has revived in recent years (Rogers *et al.*, 1948; Bould *et al.*, 1953; Greenham, 1952), the practice is generally to sow a grass–clover sward and to keep this cut down by mechanical means.

In 1950, an experiment was initiated at the Appleby Research Orchard, Nelson, to determine effects of grassing down on tree growth and production under the conditions pertaining in that district. The experiment is continuing, but this progress report gives a description of the methods and layout, and crop figures over the first six years.

Material and Methods

In 1950, a block of 35-year-old Jonathan apple trees, which had been kept continuously under clean cultivation and cover crops, was divided into three blocks. Half of each block was sown with a pasture mixture, the other half was kept under the normal system of orchard

management (i.e. cover crop in winter and clean cultivation in summer). Within each grassed or cultivated half-block there were three 4-tree plots. Each plot was separated from its neighbour by a buffer row of trees.

Seed Mixture. The following seed mixture was sown in March 1950:

	lb/ac.
Perennial ryegrass	25
Chewing's fescue	1½
Browntop	1½
White clover	3
Lotus major	2

Grass Cutting. In the grassed areas the aim has been to cut the grass when it reached a height of 4 to 5 in., the cuttings being allowed to remain where they fall. In the first two years, grass was cut with a small cutter-bar mower, but, as this was unsatisfactory, for the last four seasons a tractor-driven Hayter mower (Fig. 1) with horizontally rotating blades was used.

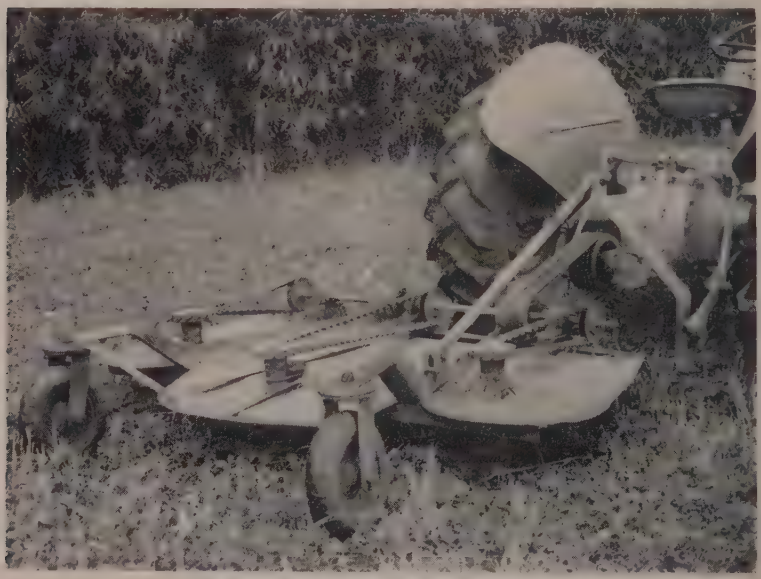

Fig. 1.—Hayter tractor-driven mower.
—*Photo: J. W. Endt.*

Tree Management. Apart from treatment differences, all trees received uniform management. No irrigation is practised in this orchard. The average annual rainfall is 38 in.

Manurial Treatments. Up to 1930, these trees had received little fertilizer. From 1930, all trees received a mixture of fertilizers each year at the following rate:

4 lb. superphosphate
2 lb. sulphate of ammonia
1 lb. sulphate (or muriate) of potash per tree.

In the spring of 1950 the following manurial treatments per tree per year were superimposed on both grass and clean-cultivation treatments:

4 lb. superphosphate plus 2 lb. sulphate of ammonia
4 lb. superphosphate plus 4 lb. sulphate of ammonia
4 lb. suplhate of ammonia

These treatments will be referred to as 4P2N, 4P4N, and 4N treatments respectively.

Each of these treatments was applied to a single plot of 4 trees in each of the three grassed and in each of the three cultivated half blocks. No other fertilizers were applied. The whole area had been receiving and continued to receive carbonate of lime at the rate of one ton per acre every fourth year.

Yield Records. Fruit from each tree was weighed at picking time. The first records were taken in 1950, before grass was sown. From 1950 to 1954, fruit was picked at commercial harvesting dates, and windfalls, which were negligible, were not recorded. In both 1955 and 1956, picking was late and the amount of fruit falling was appreciable. In the data for 1956, the weight of windfalls is included in the yield figures but no weights of windfalls are available for 1955.

In 1953, a number of trees in one of the clean-cultivated half-blocks died from attack of *Phytophthora cactorum.* Since then no records have been taken from either the remaining trees in this half-block or the trees in the corresponding grassed half-block. All records subsequently presented in this paper are only those derived from the trees in the two replications that have remained intact.

RESULTS

Sward Growth

A good germination and strike of seedlings was obtained and no difficulty has been experienced in maintaining a good grass cover in each of the blocks. Although all species sown were evident initially, the pasture soon became dominated by perennial ryegrass and white clover until, by about two years from sowing, there was little evidence of the presence of other species. Clover is still abundant in all plots.

For a variety of reasons, the objective of mowing whenever grass reached a height of 5 in. was not always achieved. The number of cuts actually made was as follows:

July 1951 — June 1952	10
July 1952 — June 1953	16
July 1953 — June 1954	12
July 1954 — June 1955	7
July 1955 — June 1956	8

In general, grass was cut approximately once a fortnight during spring and early summer, extending to once a month during late summer and autumn. Usually there was only one cut each winter.

Fruit Yields

The average crops from trees of each treatment for the years 1951 to 1956 are shown in Fig. 2. These data have been adjusted by analysis

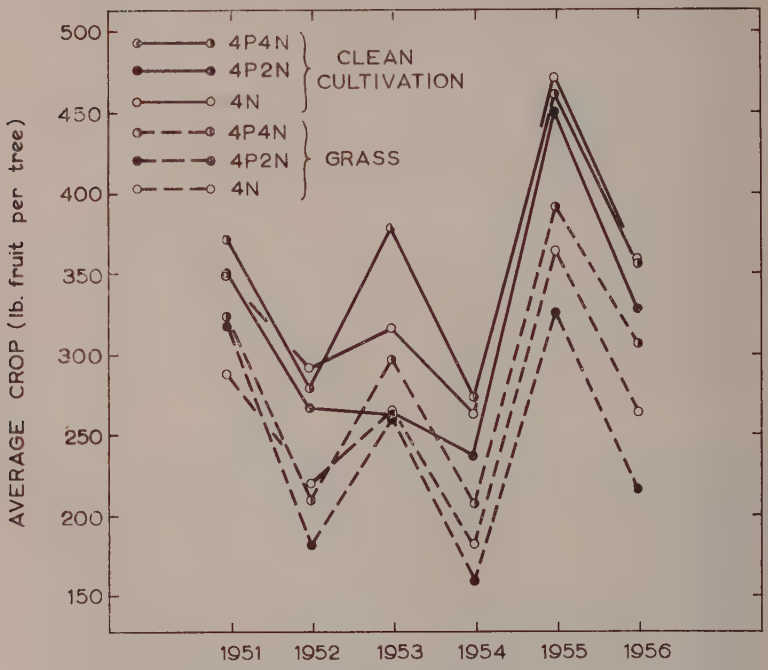

Fig. 2.—Yields from Jonathan apple trees under varying soil management and manurial treatment.

of covariance on the basis of crops produced in 1950 (before treatments were imposed) to allow for initial variation in cropping capacity. Statistical analysis of each year's results showed significant differences (at 5%) in only 1952. In this year the average yields of trees receiving each manurial treatment under clean cultivation were significantly greater than those of trees receiving the same manurial treatment under grass.

Table 1 gives the six-year average yield (adjusted as above) from each treatment.

TABLE 1. SIX-YEAR-AVERAGE WEIGHT OF FRUIT IN LB. PER TREE FOR 1951-56 INCLUSIVE

Manurial Treatment	Soil Management	
	Grass	Clean Cultivation
4P2N	242.8	315.5
4P4N	288.8	353.1
4N	263.5	341.4
Sig. Diff. 5%	25.5	
1%	34.5	

Considering these average yields it can be seen that:

1. With each manurial treatment, yields under clean cultivation are significantly higher (at 1%) than under grass.
2. With both clean cultivation and grass, yields from 4P4N manurial treatment are significantly higher (at 1%) than those from the 4P2N treatment.
3. Yields from the 4P4N treatment under grass are significantly lower (at 5%) than from the 4P2N treatment under clean cultivation.
4. Under both clean cultivation and grass, yields from 4N treatment are not significantly lower than those from 4P4N treatment.

DISCUSSION

No difficulties were experienced in establishing pasture in the orchard. Nor has there been any apparent tendency for the clover to disappear from the sward even where high dressings of sulphate of ammonia have been used. Rogers *et al.* (1948) found difficulty in retaining clovers in an orchard sward while using sulphate of ammonia, but they also pointed out that, with frequent mowing, retention of the clover under these conditions might be possible.

The frequency of mowing in the present experiment was less than originally intended. To keep the grass strictly below the specified 4 to 5 in. would certainly have required more cuts in some seasons.

Previous work* has shown that yields from this orchard are sensitive to nitrogenous fertilization. The significant increase in yield obtained with the 4P4N treatment under clean cultivation over that with the 4P2N treatment is of the order expected from the earlier work.

A noteworthy feature of the present results is the reduction in yield obtained under each grassed treatment in contrast with its corresponding treatment under clean cultivation. English work indicated that a reduction in tree vigour is likely when an orchard is grassed down, but the extent of reduction in the present work is perhaps greater and of longer duration than expected.

The supply of extra nitrogen to trees being grassed down has resulted in significantly higher yields, as can be seen from a comparison of yields from the 4P2N and 4P4N treatments under grass. Thus extra nitrogen can offset to some extent at least the reduction in yield which occurs when trees are grassed down. However, the extra nitrogen used in the present experiment (2 lb. sulphate of ammonia per tree per year) was not enough to maintain yield at the level of trees remaining under clean cultivation without extra nitrogen. In an area with soil less deficient in nitrogen it is possible that the extra nitrogen used in this experiment would have been sufficient to maintain the yield of grassed trees.

The yields from trees from which phosphate has been omitted are not significantly lower than those from trees continuing to receive phosphate. All trees used in this experiment had received substantial

* An account of the manurial experiments at the Appleby Research Orchard, Nelson, will be published as a bulletin in the *N.Z. Dep. sci. industr. Res.* Series.

quantities of phosphate for twenty years preceding initiation of this experiment, and the effects of this are still apparent.

This experiment is continuing, but this progress report should serve as a guide to the sort of response that may be expected when trees in this district are grassed down. It is probable that considerable advantages will accrue from this form of soil management, but the fact must be faced that reduction in tree yields may occur unless extra nitrogen is supplied.

Acknowledgement

Acknowledgement is due to Dr. H. R. Thompson of Applied Mathematics Laboratory, Department of Scientific and Industrial Research, who carried out the statistical analysis.

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A *Pythium* DISEASE OF PEAR TREES

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(*Received for publication, 31 October 1956*)

Summary

A species of *Pythium* pathogenic to pears is described. This fungus attacks phloem of trunks, leaders, and occasionally small branches. Affected parts of trees fail to make new shoot growth and shed leaves prematurely. Fruits remain small and hard. Affected limbs die, often being attacked secondarily by *Diaporthe perniciosa, Stereum purpureum,* and *Coriolus versicolor;* within 3 to 10 years the tree dies.

Most varieties of pear are affected. The fungus has also been isolated twice from apple trees.

Orchard incidence may be as high as 20% although the disease is only serious as yet in the Te Kauwhata district.

Suggested control measures are avoidance of root wounding and removal of badly affected leaders or trees. It might be beneficial to treat the soil of infected sites before replanting unless trees other than pears or apples are used.

Introduction

In spite of careful orchard practice a steady deterioration has been going on in certain Te Kauwhata pear orchards for at least 16 years. Trees have slowly died and replacement trees have also been affected in some instances. Prior to the commencement of this work there had been no satisfactory explanation for the decline. It was suspected, however, that large stem cankers on some of the affected trees might have contributed to the condition. During examination of a canker on a piece of stem taken from an affected tree a narrow band of orange discolouration was noticed in the inner phloem of otherwise healthy bark beyond the canker. From the discoloured tissue a phycomycetous fungus was isolated which appears to be a hitherto undescribed species of *Pythium.*

Perotti (1947) and Refatti (1948) describe symptoms of decline affecting mature pear trees in northern Italy. While no cause for the decline has been discovered as yet, outward symptoms are similar in many respects to those of the New Zealand *Pythium* disease.

Symptoms

The fungus causes a distinct orange staining, usually of one layer of the inner phloem (Figs 1, 2). It is apparently prevented from passing easily to other annual layers by the almost continuous zones of stone cells separating them. Sometimes infection reaches the cambium, causing growth to cease in this region. Discoloured phloem may be present in narrow or wide bands or encircle the trunk. It is often seen in roots near the trunk and in all cases examined infection appears to have originated from entry through cultivation injuries to roots. Vertically, infection may reach only to the crotch although usually it can be traced up leaders to a height of several feet. Occasionally it has been found to extend out to twigs. In older-affected portions of phloem the orange colour deepens and tissue eventually turns black. At this stage the pathogen is seldom recovered by isolation.

Fig. 1

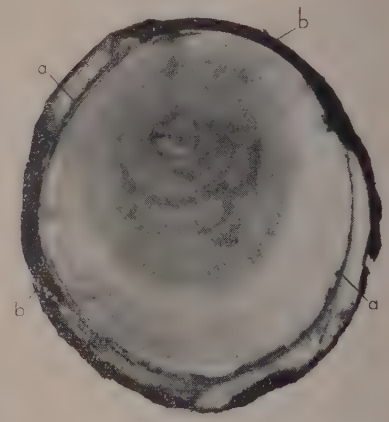

Fig. 2

Fig. 1.—Pear limb with part of bark and wood removed to show sections through (a) stained layer of inner phloem (b) bark canker.

Fig. 2.—Transverse section through pear limb to show (a) stained layer of inner phloem (b) bark cankers.

Fig. 3.—Healthy pear tree showing vigorous upright shoots of current season's growth on periphery of crown.

Fig. 4.—Affected pear tree showing pale foliage and lack of new shoot growth (cf. Fig. 3).

Fig. 5.—Pear limb showing bark cankers (right side) and section through bark on margin of canker. Discoloured inner phloem shows on left side of section.

Affected leaders are characterized by a lack of shoot growth (Figs. 3, 4) and by excessive spur development. Leaves may be smaller than normal and pale in colour, and show premature development of autumn tints and early leaf fall; some leaf scorch may be evident. Fruit on slightly affected leaders may ripen prematurely, but on more severely affected

branches fruit is typically small and remains hard. Owing to sparseness of foliage, fruit sometimes suffers from sunscald. Twig die-back is common, and eventually affected leaders die, death frequently being hastened by secondary infection with *Stereum purpureum* Pers. or *Coriolus versicolor* (Linne ex Fr.) Quel. One or all leaders of a tree may be affected at once. Growers report that death of leaders typically occurs about three years after first signs of deterioration occur. Death of trees may take anything from three to ten years. Although deterioration is slow, no cases of recovery have been reported.

Commonly, but by no means invariably, badly infected leaders may have bark cankers (Fig. 5) from which the weak parasite *Diaporthe perniciosa* Marchal can usually be isolated. The cankers extend right to the wood and so differ from the relatively superficial scale-bark symptoms with which *D. perniciosa* is normally associated on pears. Their extensiveness is probably due to the greater susceptibility to *D. perniciosa* of *Pythium*-infected branches rather than to the latter fungus breaking out to the exterior of the stems and being overrun by the weaker parasite. Cankers may be quite small or extend continuously or as a succession of cankers for several feet. Cankers are depressed, usually smooth, and separated from healthy tissue by a fissure.

Isolation

Initially, isolations from diseased tissue were made by aseptically removing pieces of discoloured phloem, or surface-sterilizing pieces of diseased tissue by rinsing for 30 sec. with a 10–25% solution of commercial sodium hypochlorite, and plating in water agar. Isolation of this fungus by either technique was inconsistent and often negative. A later modification of the isolation technique increased the recovery rate by several times. Small chips of diseased phloem were held in muslin bags under running water to leach for 30 min. and then plated directly or, preferably, after rinsing in sodium hypochlorite solution. Leaching appeared to remove a fungistatic factor which otherwise developed in detached bark tissue.

Pathogenicity

Portions of cultures of the fungus grown on potato dextrose agar were inserted under triangular flaps of bark partly lifted from the bases of the stems of 2-year-old dormant pear trees below and at ground level. Wounds were covered with cellophane adhesive tape and petroleum grafting wax. Check trees were similarly wounded but not inoculated. Six months later all inoculated trees showed an orange discolouration of the inner phloem for several inches above the wounds, similar in appearance to internal symptoms on the original specimens. The fungus was recovered from this tissue in all cases. Checks showed no phloem symptoms.

Description of the Pathogen

Hyphae are non-septate when young, measuring 1.5–6.0μ in diameter. Sporangia are smooth and thin walled, are borne terminally, and are spherical or obovate in shape or more commonly oblong or reniform with rounded ends; their size ranges from 15–48 $\times$ 12–30μ (average 33 $\times$ 21μ). Oogonia are terminal, smooth, thin-walled, spherical to subspherical, and 11–18μ (average 14.5μ) in diameter.

Oospores are plerotic, smooth, single, with a moderately thickened wall, 10–16μ (average 13μ) in diameter. Antheridia are borne on long or short branches; they may or may not be cut off by a septum, are monoclinous or diclinous, and are small and clavate, 2–4 × 6–9μ in size. There are 1–3 antheridia to each oogonium, contact being apical.

HOST RANGE

Pears. The pathogen was isolated from trees of the following pear varieties: William's Bon Chretien (Bartlett), P. Barry, Packham's Triumph, Beurre Bosc, Winter Cole, Keiffer. All trees showed typical symptoms.

Apples. Only two cases of infection have been found, the fungus being isolated from a large Jonathan tree adjacent to a block of infected pears, and a similarly sited Gravenstein on another orchard, both at Te Kauwhata. Only the latter showed signs of debility at the time of examination. In both instances infection appeared to have entered through cultivation injuries on major roots.

DISTRIBUTION AND ECONOMIC IMPORTANCE

The original specimen came from an isolated tree on a farm in the Te Kauwhata district. Surveys subsequently carried out by the Horticulture Division showed that the disease is more serious in this district than in any other major pear-growing area of New Zealand.

The disease was recorded from five of the six major Te Kauwhata pear orchards, the percentage of infected trees being as high as 20 on one property. Tree deterioration was reported to have been a feature of these orchards for 16 years prior to this investigation.

Incidence in other districts:

Henderson, 10 orchards affected, *Pythium* isolated from 1–8% of trees with exception of one small orchard with 15% infection (5 trees of 32).

Huapai, 1 orchard, 3% trees affected.

Hastings, 2 orchards, under 1% trees affected.

Pythium has not been found on pears in Nelson, Marlborough, Canterbury, or Central Otago.

CONTROL MEASURES

The pathogen is apparently soil borne and enters trees through cultivation wounds on roots. Care should be exercised in orchard cultivation. Grassing down should be advantageous in removing the risk of cultivation injury to roots and in preventing spread of infected soil. It is possible that soil treatment with fungicides or fumigants would be beneficial before replanting sites from which infected trees have been removed. An alternative would be to replant infected sites with other types of fruit.

No treatment can be offered for infected trees. It is suggested that as there appears to be no risk of infection spreading from aerial parts of trees, affected leaders or trees be retained so long as fruit quality and

quantity are reasonable. Dead or dying branches should be removed and wounds treated to prevent silver-leaf infection.

Acknowledgements

The author wishes to thank Mr. C. E. K. Fuller, formerly Department of Agriculture, for submitting the first specimen and for assistance on several occasions in the field; officers of the Department of Agriculture, who carried out surveys in the main pear-growing districts; and many orchardists for their co-operation.

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CROWN RUST OF RYEGRASS

By I. A. M. Cruickshank*, Plant Diseases Division, Department of Scientific and Industrial Research, Auckland

(*Received for publication, 6 August 1956*)

Summary

It is demonstrated that perennial ryegrass infected with crown rust (*Puccinia coronata* var. *lolii*) is unpalatable to sheep.

Seventy-two strains of perennial, Italian, and short-rotation ryegrass, and hybrids between perennial and short-rotation ryegrass, were tested for reaction towards a New Zealand-wide collection of crown rust. Short-rotation ryegrass was highly resistant and perennial ryegrass highly susceptible. Italian ryegrass and the short-rotation × perennial hybrids were intermediate in reaction towards crown rust.

The significance of these tests in relationship to breeding and selection of ryegrass is briefly discussed.

Introduction

Crown rust (*Puccinia coronata* Corda var. *lolii* Brown) was first recorded on perennial ryegrass (*Lolium perenne* L.) and Italian ryegrass (*L. multiflorum* Lam.) in New Zealand by Cunningham (1922). This disease is widespread throughout the pastures of New Zealand. The intensity of infection varies with district and season. Severe infection is more common in the North Island than in the South Island, and usually occurs in the late summer and early autumn of a warm dry year. Under these conditions it may be an important factor limiting green-feed production at a time of the year when feed is normally at a minimum owing to other causes. Field observations suggest that, in addition to the effect of crown rust on yield, rust-infected forage may affect the health of ruminants by unpalatability or toxicity.

Economic control of crown rust would appear to be only possible by the breeding of a suitable range of resistant ryegrass strains. This paper presents a note on the palatability of rust-infected perennial ryegrass, and results of crown rust reaction, under glasshouse conditions, on a wide range of naturally occurring ryegrass strains and hybrid ryegrass progenies.

Experimental

Grazing Trial

Crown-rust notes were taken at Grasslands Division, Palmerston North, in February 1956 on a block of 675 spaced perennial ryegrass plants originating from 69 crosses of 13 parent plants—one replication of a block of 3,600 plants. An 0-4 scale of intensity of rust infection was used.

A note on growth (0-5 scale) was taken on each of the 675 individual plants; 145 sheep were grazed on the whole block (3,600 plants) and removed after five hours; the growth of the plants was again estimated and the amount of leaf eaten was calculated as the

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difference in growth before and after grazing. The data are summarized in Table 1.

TABLE 1. EFFECT OF CROWN-RUST INFECTION ON PALATABILITY TO SHEEP

Rust Scale*	No. of Plants	Mean †Growth per Plant Before Grazing	Total Growth	Amount Eaten	Percentage Eaten
0	26	3.23	84	83	99
1	109	3.00	327	270	83
2	174	3.07	534	294	55
3	247	2.71	670	119	18
4	119	2.08	247	2	1

* 0 = no pustules: 1 = few pustules: 2 = moderate rust: 3 = severe rust: 4 = severe rust with leaves withered.

† Amount of growth assessed on a productivity scale of 0-5.

Rust Reaction of Ryegrass Strains and Progenies

Individual physiologic rust races as such, within *P. coronata* f. *lolii* were disregarded. During the autumn of 1955, crown-rust-infected ryegrass turf was collected from the following districts: Alexandra, Ashburton, Auckland, Balclutha, Blenheim, Christchurch, Dannevirke, Dargaville, Dunedin, Gisborne, Hastings, Hawera, Kaikohe, Kaitaia, Levin, Lincoln, Matamata, Masterton, Nelson, Pukekohe, Rangiora, Rotorua, Tauranga, Taumaranui, Te Awamutu, Te Kuiti, Thames, Timaru, Waimate, Wairoa, Waitemata, Wallaceville, Whakatane, and Whangarei, Uredospore samples were collected from them. Where necessary, the original material was re-inoculated in order to obtain sufficient spores for tests and storage.

Seventy-two strains of perennial, Italian, and short-rotation ryegrass, and hybrids between perennial and short-rotation ryegrass (Table 2) were tested in two trials under glasshouse conditions (5 pot-replications of 10 plants per strain). The plants tillered well and were cut to a height of 5-6 in. prior to inoculation. All strains were tested in the first trial. Only those showing some resistance were included in the second trial.

The inoculum used was a mixture of approximately equal aliquots of each of the 144 uredospore collections from the 34 districts listed above. Inoculations were carried out using a compressed-air (5 lb/sq. in.) all-glass semi-micro duster (Tervet and Cassel, 1950), otherwise the technique followed was similar to that previously described (Cruickshank, 1950).

Rust reaction was assessed on the basis of the mean pustule type classified as follows:

0-0 no uredia, chlorotic flecks may be present (highly resistant).

1-2 small uredia with large chlorotic or necrotic halos (resistant).

3-4 large uredia with only faint chlorosis (susceptible).

RESULTS

Results of the first inoculation trial are presented in Table 3. Results of the second trial confirmed the data presented.

TABLE 2. LIST OF RYEGRASS STRAINS

Grasslands Division Accession No.	Source of Origin
Perennial	
E 1346	Southland, uncertified
A 1748	Dutch pasture strain
A 1749	Dutch hay strain
A 1750	Welsh Pl. Breed. Sta. S23
A 1751	Welsh Pl. Breed. Sta. S24
A 1752	Welsh Pl. Breed. Sta. S101
A 1754	Danish Roskilde
A 1756	Garton's England
A 1758	Danish Hunsballe
A 1898	Devon Eaver
A 1899	Irish
A 1933-39	1955 Nucleus Isolation progenies
A 2009	Spain
A 2010	Spain
A 2015-17	N. Canterbury, non pedigree
A 2032-33	Hawke's Bay, non pedigree
A 2034	Oregon, U.S.A.
Italian	
E 1344	Waiuku, uncertified
E 1345	Tirau, uncertified
B 1996	N.Z. Certified Commercial
B 1997	Victoria, Australia
B 1998	South Australia
B 2021	Wimmera ryegrass
B 2022	Welsh Pl. Breed. Sta. S22
Short-rotation	
E 1335 — E 1343	1955 Nucleus Isolation progenies
Perennial x *Short-rotation*	
N 153 — N 287	Progenies from interpollination in polycross block

TABLE 3. REACTION OF RYEGRASS STRAINS TO CROWN RUST

Accession No.	Percentage in Each Pustule Class			Accession No.	Percentage in Each Pustule Class		
	0-0	1-2	3-4		0-0	1-2	3-4
Perennial				B.2008	42.0	6.0	52.0
				B.2009	49.0	9.0	42.0
E.1346	2.2	15.2	82.6	B.2010	54.0	11.0	35.0
A.1748	14.3	0	85.7	B.2011	40.0	18.0	42.0
A.1749	38.8	39.0	22.2	B.2012	30.0	17.0	53.0
A.1750	9.4	16.8	73.8	B.2018	37.0	5.0	58.0
A.1751	0	0	100.0	B.2021	61.0	6.0	33.0
A.1752	0	0	100.0	B.2022	9.0	59.0	32.0
A.1754	34.2	7.8	58.0	Average	35.0	17.6	47.5
A.1756	19.0	13.6	67.4				
A.1758	16.6	2.0	81.4	*Short-rotation*			
A.1898	0	2.0	98.0				
A.1899	0	2.0	98.0	E.1335	90.0	10.0	0
A.1933	2.0	4.0	94.0	E.1336	72.0	18.0	10.0
A.1934	12.0	16.0	72.0	E.1337	71.0	24.5	4.5
A.1935	4.4	13.0	82.6	E.1338	82.0	18.0	0
A.1936	0	10	90.0	E.1339	75.0	23.0	2.0
A.1937	8.5	16.7	74.8	E.1340	63.0	32.0	5.0
A.1938	13.6	14.4	72.0	E.1341	81.0	19.0	0
A.1939	5.0	25.0	70.0	E.1342	58.0	25.0	17.0
A.2009	4.0	38.0	58.0	E.1343	88.0	6.0	6.0
A.2010	2.6	10.0	87.4	Average	68.9	25.9	5.2
A.2015	0	23.0	77.0				
A.2016	2.0	4.0	94.0	*Perennial* x *Short-rotation*			
A.2017	3.0	19.5	77.5				
A.2032	0	0	100.0	N.152	60.0	22.0	18.0
A.2033	6.0	16.0	78.0	N.158	50.0	32.0	18.0
A.2034	4.0	6.0	90.0	N.164	43.0	43.0	14.0
Average	7.5	11.6	80.9	N.177	39.0	26.0	35.0
				N.179	23.0	42.0	35.0
Italian				N.183	35.0	45.0	20.0
				N.195	58.0	18.0	24.0
E.1344	39.0	18.0	43.0	N.204	61.0	36.0	3.0
E.1345	27.0	20.5	52.5	N.206	16.0	44.0	40.0
B.1996	20.0	6.0	74.0	N.219	33.0	57.0	10.0
B.1997	42.0	12.0	46.0	N.221	45.0	45.0	10.0
B.1998	18.0	6.0	76.0	N.225	46.0	51.0	3.0
B.2002	37.0	22.0	41.0	N.236	39.0	34.0	27.0
B.2003	38.0	38.0	24.0	N.252	47.0	38.0	15.0
B.2004	39.0	7.0	54.0	N.284	53.0	21.0	26.0
B.2005	30.0	19.0	51.0	N.287	58.0	19.0	23.0
B.2006	38.0	33.0	29.0	Average	44.1	35.8	20.1
B.2007	15.0	21.0	64.0				

DISCUSSION

Sampson and Western (1941) stated that crown rust was responsible for considerable defoliation of ryegrass, and that herbage was rendered unpalatable to stock, but they gave no experimental evidence to support this. Results presented above closely confirm their statement, at least under conditions where sheep have a choice of forage. The mean growth per plant is also reduced as the rust infection becomes more severe, but this may not be a simple relationship under field conditions as plant vigour and maturity are also involved.

Crown-rust reaction varied considerably both within and between strains and progenies. No ryegrass strain tested was immune to crown rust but with few exceptions the strains or progenies within the groups listed fell into distinct reaction classes. Perennial-ryegrass strains were highly susceptible, with the exception of one strain of Dutch origin (A.1749). Short-rotation ryegrass progenies were highly resistant. These progenies are the result of hybridization of perennial × Italian ryegrass and subsequent selection at Grasslands Division where one of the factors considered in selection was resistance to crown rust. Italian ryegrass strains and the perennial × short-rotation progenies gave an intermediate rust reaction. Although both these groups have been selected for rust resistance, this selection has not been as effective as in the case of short-rotation ryegrass, perhaps because Italian ryegrass is often not vegetatively vigorous when rust infection is severe, and because few selections have been possible in the short time the perennial × short-rotation breeding project has been in progress.

All selection for crown-rust resistance in ryegrass in New Zealand has been based on natural field infection at Palmerston North. Present results indicate that this work has been valuable and that selections made on this basis react similarly when tested with rust collections of much wider origin.

CONCLUSIONS

It is concluded, first that an important aspect of crown-rust infection is that, in addition to reducing vigour and yield, rust also renders ryegrass unpalatable to sheep. Secondly, there appears to be considerable variation within strains and progenies, which could be utilized in the selection of more resistant types especially in the perennial × short-rotation hybrids. Field selection combined with glasshouse testing with a wider range of rust collections or rust races appears to be the most likely method of increasing the resistance of ryegrass to crown rust.

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TRIALS OF FRUIT-SETTING SPRAYS ON GLASSHOUSE TOMATOES

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Summary

In tests with various fruit-setting sprays on tomatoes grown in unheated glasshouses in Auckland during 1950 and 1952, betanaphthoxyacetic acid, parachlorophenoxyacetic acid and certain commercial formulations increased yields and mean fruit weights on early crops which developed under adverse weather conditions.

Sprays of betanaphthoxypropionic acid at 5 and 10 p.p.m., and one commercial formulation were unsatisfactory.

Compared with checks most treatments gave heavier early pickings.

Quality of fruit set by effective treatments was good in spite of some slight hollowing.

Introduction

Since Gustafson (1936) first induced parthenocarpic development of tomato fruits with betaindolylacetic acid, a wide range of growth substances have been found effective for this purpose. Today, growth-substance sprays are used widely both overseas and in New Zealand for supplementing fruit set in glasshouse tomato crops grown during winter or early spring. At this time of the year, because of low temperatures and poor light conditions, much of the pollen produced is sterile, pollination is inadequate, and yields of fruit from lower trusses are generally unsatisfactory.

Results of preliminary tests of various commercial fruit-setting formulations on glasshouse tomatoes in Auckland were reported by Woodhead and Jacks (1951). Their demonstration of the ineffectiveness of one proprietary formulation, and reports of other unsatisfactory sprays, led to further trials of several growth substances in various formulations. An account of these trials is given below.

Methods and Results

Chemicals used in these experiments were: betanaphthoxyacetic acid (NOA), parachlorophenoxyacetic acid (CPOA) and betanaphthoxy-propionic acid (NOP).

Commercial products used were: Fulset, Murphy Tomato Set, Tomatone, and Fruitone.

Potentate tomato plants grown in beds in unheated glasshouses were used. Each treatment was carried out on 5 or 6 rows. Sprays were applied to the basal three trusses of each plant by small hand sprayers.

Unformulated chemicals were made up by dilution from a concentrated alcoholic solution. Commercial proprietary sprays were prepared as directed.

Weights and numbers of fruit harvested from sprayed trusses were recorded and from these a mean fruit weight was calculated. Observations of fruit quality were also made.

1950 Avondale Trial

Plants were set out in the houses on 4 June 1950. Commencing 21 August, applications were made when three-quarters of the flowers on each truss had opened.

Fine days and cool night temperatures predominated during flowering of basal trusses. Fruit did not set freely on the check plants. Conditions became milder during development of the third truss however and increased natural set was noted.

Details of treatments and results are given in Table 1.

TABLE 1. EFFECT OF FRUIT-SETTING SPRAYS ON GLASSHOUSE POTENTATE TOMATOES. AVONDALE, 1950

Treatment	Mean Yield (lb.) of Three Trusses/13 Plants	Mean Fruit Weight (oz.)	Mean Yield (lb.) in First 7 Days/13 Plants
Check	26.6	1.50	2.9
Betanaphthoxyacetic acid (NOA) 30 p.p.m.	32.9	1.78	8.2
„ „ 50 „	36.5	1.87	8.4
Parachlorophenoxyacetic acid (CPOA) 10 p.p.m.	36.5	1.90	8.7
Betanaphthoxypropionic acid (NOP) 5 p.p.m.	26.8	1.49	5.2
„ „ 10 p.p.m.	29.2	1.55	7.7
Fulset (diluted 20:1) (Active ingredient betanaphthoxyacetic acid at 60 p.p.m.)	35.4	1.87	10.3
Fruitone 1 oz./12½ gal.	26.6	1.52	5.5
Sig. diff. 5%	3.3	0.16	2.7
1%	4.5	0.22	3.7

1952 Oratia Trial

Plants were set out during the first week of June and basal trusses were ready to treat on 15 August 1952. Two methods of spraying were compared, a single spray and a double spray. The single-spray treatment was carried out when the majority of flowers on the truss had opened. The first application of the double-spray treatment was made when approximately a third to a half of the flowers on the truss were fully open, and the second 7-10 days later when the remaining buds had opened.

Details of treatment and results are given in Table 2. Check plants were shaken every 2-3 days to assist pollination. Intermittent periods of rain and cool nights over the flowering periods made conditions unfavourable and check plants did not set fruit freely.

Effect on Yield and Fruit Size

Under the adverse weather conditions, sprays of NOA, CPOA, and certain of the commercial formulations, increased yields and mean

TABLE 2. EFFECTS OF VARIOUS COMMERCIAL FRUIT-SETTING FORMULATIONS ON GLASSHOUSE POTENTATE TOMATOES, ORATIA, 1952

Treatment	Mean Yield (lb.) of Three Trusses/9 Plants	Mean Fruit Weight (oz.)	Mean Yield (lb.) in First 14 Days/9 Plants
Check—hand shaken	33.4	1.95	3.6
Murphy Tomato Set (diluted 100:1) Double spray (active ingredient, betanaphthoxyacetic acid 40 p.p.m.)	46.5	2.86	6.9
Fulset (diluted 20:1) single spray (active ingredient betanaphthoxyacetic acid 60 p.p.m.)	42.6	2.49	3.8
Fulset, (diluted 20:1) double spray	46.1	2.97	9.2
Fulset, (diluted 30:1) double spray (active ingredient at 40 p.p.m.)	43.8	2.84	8.2
Tomatone (diluted 150:1) single spray (active ingredient chlorinated phenoxyacetic acid at 10 p.p.m.)	45.6	2.53	3.9
Tomatone (diluted 150:1) double spray	46.0	2.83	9.1
Sig. diff. 5%	4.4	0.21	2.3
1%	5.9	0.29	3.1

fruit weight. Increases in yield from the three basal trusses ranged up to 39% compared with checks. Treatments were effective in both seasons.

Sprays of NOP at 5 and 10 p.p.m. and Fruitone proved unsatisfactory.

In 1952, the double spray of Fulset at a dilution of 20:1 increased yields more than the single spray at the same concentration but the difference was not significant at the 5% level. The double spray at 20:1 similarly increased yields more than the double spray at 30:1 but the difference was again not significant at the 5% level. Double sprays of Fulset and Tomatone gave a significantly greater mean fruit weight than the corresponding single spray.

Effect on Earliness of Picking

A striking result of most of the effective treatments was the marked increase in heaviness of early pickings. For example, with certain treatments in 1952, over ½ lb. more fruit per plant was picked from sprayed rows than from check rows in the first 14 days of harvesting. In 1952, heavier early pickings were obtained from rows receiving the double sprays than from those receiving a single spray.

Effect on Fruit Quality

The quality of fruit produced by effective treatments was good, although slight hollowing occurred in both seasons. In 1952, double

sprays appeared to cause slightly more hollowing than single sprays. However, in no instance was this defect of economic significance. Many of the fruits were seedless and showed a characteristic greenish colouration of pulp tissue.

Basal trusses of check plants and those receiving sprays of NOP and Fruitone produced many small solid fruits. With increased natural set, the second and third trusses of plants receiving these treatments set a larger proportion of fully seeded normal fruits.

DISCUSSION

The effectiveness of various plant-growth-substance sprays for supplementing natural fruit set under unfavourable conditions in unheated commercial glasshouses is well shown in these trials. Results indicate also that certain of the sprays tested, viz. betanaphthoxypropionic acid 5 and 10 p.p.m. and Fruitone are unsuitable as fruit-setting agents. Zimmerman and Hitchcock (1944) and Wain (1949) considered betanaphthoxypropionic acid an effective fruit-setting spray when used at the much higher concentration of 100 p.p.m.

Results of these and other tests have led to the publication of lists of recommended fruit-setting formulations (Bollard 1955).

It has often been shown that the use of growth-substance fruit-setting sprays gives heavier early yields from crops grown under unfavourable setting conditions. This effect was confirmed in both seasons, but was evident in 1952 only with the double-spray treatments. With the single sprays, effectiveness in increasing early yields seemed to depend on time of application, for in 1950 sprays were applied at a slightly earlier stage of flowering than in 1952. Similarly, the heavier early pickings obtained with double sprays in 1952 can probably be explained by the differences in time at which the first sprays were applied.

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THE RACHITOGENIC EFFECT OF VITAMIN A

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Summary

It has been shown that the dead brown leaves of pastures contain appreciable amounts of vitamin D whereas the green leaves contain a rachitogenic factor.

A rachitogenic factor has been isolated from greenfeed and identified as carotene.

The factor is more properly vitamin A since equivalent amounts of vitamin A and carotene have the same rachitogenic potency.

Vitamin A probably has no direct inhibitory effect on vitamin D but appears to exert its rachitogenic influence in some other way, possibly by its local action at the site of bone formation.

Introduction

Osteoporotic or rickets-like conditions in New Zealand sheep have been reported by Reid and Aston (1910), Reakes (1912), and Hopkirk (1935). Leslie (1935) expressed the opinion that ovine rickets was the most common disease of growing lambs in New Zealand, appearing during autumn and winter in animals grazing upon green-feed cereal crops, Italian ryegrass, rape, or kale. His diagnosis of the disease was based upon observations that rugged sheep were more prone to an outbreak than uncovered animals in the same flock, that rapid recovery often followed shearing, and that beneficial effects resulted from the administration of cod-liver oil.

It was pointed out by Fitch (1943) that rickets or rickets-like conditions in sheep had been frequently reported in many parts of the world, but that, in most cases, the disease had not been confirmed histologically. In the cases which had been so examined, the condition was usually some other abnormality resulting in osteoporosis. The only confirmed report of true rickets in sheep which he found in an exhaustive search of the literature was that of Marek and Wellmann (1931), who described cases occurring on two properties in Hungary. Fitch thoroughly examined the clinical, post-mortem, and histological features of the disease as it occurred in New Zealand lambs grazing upon winter greenfeed and demonstrated that the overall picture was one of true rickets. The evidence he produced indicated that a simple dietary deficiency of calcium and/or phosphorus was not involved. He drew attention to the fact that though the relative freedom from rickets of man and other animals in New Zealand had led to the assumption that solar ultraviolet radiation provided sufficient vitamin D to prevent the disease, the regions where ovine rickets most commonly occurred in this country lay within the latitudes described as dangerous by Tisdal and Brown (1929). This consideration, together with the fact that in none of the outbreaks investigated was any vitamin D rich supplement available to the animals and also the direct experimental evidence of Andrews and Cunningham (1946) that solar irradiation at Wallaceville was inadequate for sheep during the winter months, indicated that the disease could be attributed to a simple vitamin D deficiency. In support of this hypothesis,

there was a marked fall in both calcium and phosphorus levels of the blood of affected animals which many workers had demonstrated in avitaminosis D. While pointing out that rickets in sheep as it occurred in New Zealand could reasonably be attributed to a vitamin D deficiency, this worker drew attention to the possibility of the presence of some 'phosphorus-immobilizing substance' in the feed. Indicating such a possibility were the facts that rickets was a disease uncommon in other animals in this country and that it was apparently rare in sheep raised in far less sunny latitudes than New Zealand.

With the object in mind of treating or preventing the development of ovine rickets at reasonable cost and economy of labour, Fitch (1944) demonstrated the presence of an appreciable amount of vitamin D in the blood sera of lambs wintered on green oats, 74 days after receiving subcutaneously 20 mg. of calciferol per head. In the same flock no vitamin D was detected in the sera either of untreated animals or of a cod-liver-oil supplemented group which received weekly doses approximately equivalent to 450 I.U. vitamin D per head daily over a period of nearly four months. In a field trial, Fitch and Ewer (1944) showed that the incidence of ovine rickets on greenfeed oats was greatly reduced and a marked improvement in growth rate was obtained by dosing lambs once a week with 30 ml. of cod-liver oil per head. No antirachitic effect was observed when a supplement of 1 oz. of bone flour was dosed weekly, the animals on this treatment being just as rachitic as those of a control group which received no supplement. The sample of cod-liver oil used in this trial was not assayed, but the equivalent daily vitamin D dose of the group receiving this supplement was assumed to be in the region of 300 I.U. per head. This dose, which was not entirely effective in preventing the disease, seemed appreciably greater than the daily requirement of the growing sheep estimated as 180 I.U. per 100 lb. live weight (Andrews and Cunningham (1946)).

From the results obtained by grazing lambs on winter fodder crops during the period 1943–46, Ewer and Bartrum (1948) came to the following conclusions: Rickets occurs with the greatest frequency and severity when lambs are wintered on green cereal crops. Only occasionally do cases of rickets occur on turnips, chou moellier, and pasture, while Italian ryegrass is intermediate in its rickets-producing properties. An outbreak of the disease was more likely in a season that favoured good winter growth of pasture. They demonstrated that calciferol given to a young sheep at the beginning of winter as a single massive dose (1,000,000 I.U.) was entirely effective in treating or preventing rickets and that, providing the quality of the feed was high, the treated animal maintained a good growth rate throughout the winter. Analyses of the most rachitogenic feeds indicated that the intake of both calcium and phosphorus was adequate for the grazing animal. These workers were of the opinion that a low vitamin D status was of less importance as a causative factor than some specific principle that interfered with phosphorus metabolism and was present in the highest concentrations in green cereal plants.

The development of rickets in both sheep and guinea pigs fed on green oats grown in England has been reported by Ewer (1950). From the results of a vitamin D assay of the greenfeed it was estimated that the lambs involved were consuming in the feed approximately 700 I.U. vitamin D daily per head.

The object of this investigation was to determine whether a rachitogenic factor was present in greenfeeds and if so to determine its nature and distribution and study its physiological effects. Short accounts of some of the results presented in this paper have been reported elsewhere (Grant 1951, 1953, 1954) and many of the observations have been confirmed independently by the work of Weits (1952, 1954) who demonstrated the presence of the rachitogenic factor in the unsaponifiable fraction of hay fat.

Methods

The albino Glaxo strain of Wistar rats was used to provide the test animals throughout the investigation. The animals were from a stock on a ration low in vitamin D and were taken as weanling rats 38–42 g. in weight and approximately three weeks old.

The basal ration employed in all tests and assays was the McCollum 3143 rachitogenic diet. In each test the number of animals used varied with the supply available, and each group had a similar litter constitution. After a 28-day trial period, the animals were slaughtered and the percentage bone ash in the dry fat-free femurs determined as in vitamin D assay procedure.

Treatment of pastures for test or assay purposes consisted in drying the freshly cut material in a grass drier at 35-40°c., reducing the product to powder in a hammer mill and extracting the fat-soluble material by allowing cold chloroform to percolate through the powder packed evenly into a tube. The solvent was removed under reduced pressure at 40°c. and the resulting extract dosed to the test animals in solution in olive oil. The same amount of olive oil was dosed to the animals of all groups in any trial.

The alumina employed in the chromatographic fractionation of pasture extracts was recovered from a stock of residues by ashing at 550-600°c. until it was thoroughly bleached, treating with hot distilled water and reactivating at 250°c. for four hours. The product, which was neutral in reaction, was used and recovered repeatedly during the investigation.

Packing of the absorbent into the fractionating columns was carried out by a method which was rapid and gave more satisfactory results than the conventional method in our inexperienced hands. The method, which was devised in this laboratory, is described here in some detail since no report of a similar packing technique has been brought to the notice of the authors. The columns were made from soft glass tubing drawn out to a small bore at one end which was plugged with cotton wool. On to the plug was poured some alumina which had been deactivated in water and dried at 50°c., sufficient in amount to fill the tube to a point just above the beginning of the constriction. This was tamped down to produce a firm foundation for the activated alumina used to make up the column. The tamping rod used consisted of a flat metal disc fixed horizontally at its centre to a long narrow metal operating rod. The disc, which was somewhat smaller in diameter than the bore of the tube, so that it could be moved freely up and down, was drilled with a large number of holes each $\frac{1}{16}$ in. in diameter close together and evenly distributed over its surface. The upper end of the operating rod was lightly clamped during the packing operation so that appreciable movement was restricted to rotation and the free up and down tamping

motion. Uniform packing of the column was accomplished by inserting the rod with the disc at the bottom of the tube, pouring in the activated alumina above the disc to a depth of several centimetres then operating the rod with a simultaneous rotating and tamping motion so that the disc gradually rose as the absorbent sifted through the holes and was packed below it. As the disc approached the surface more alumina was added and the operation continued until the desired length of column was obtained. The rod was then removed from the tube and to it was attached a circle of filter paper of the same diameter as the disc and with a hole cut at its centre no larger than the diameter of the operating rod. The holes of the disc were thus covered by the filter paper and the rod placed back on to the column. The column surface was again lightly tamped and then with some downward pressure applied was firmly clamped into position. The liquid to be fractionated was carefully poured down the operating rod. The advantages of this procedure lay not only in the fact that even packing was greatly facilitated but with the filter paper in place solvent could be added with little danger of disturbing the surface of the column, and the slight pressure exerted by the rod when firmly clamped in its final position prevented the troublesome cracking of the column often experienced when a low-boiling-point liquid is used as the solvent or when solvents are changed for the purpose of zone development.

RESULTS

(1) *Vitamin D Assays on Pasture Grown in Different Seasons of the Year*

From the finding of Andrews and Cunningham (1946) that all the available winter sunshine at this station was not sufficient to prevent rickets in young sheep on a vitamin D low ration it seemed possible

TABLE 1. VITAMIN D CONTENT OF PASTURES AT DIFFERENT SEASONS OF THE YEAR

Experiment	Pasture Crop	Season of Year	Vitamin D Content in I.U./100 g. D.M.
1	Green feed oats on which sheep had developed rickets	Winter	70
2	Green oats 4–6 in. high	Winter	Negligible
3	Permanent pasture (mainly perennial ryegrass)	Winter	180
4	Green oats 4–6 in. high	Summer	Negligible
5	Permanent pasture (perennial ryegrass and white clover)	Summer	80
6	Permanent pasture (white clover and perennial ryegrass)	Spring	Negligible
7	Permanent pasture (mainly perennial ryegrass)	Autumn	Negligible
8	Italian ryegrass	Autumn	Negligible

that an inadequate vitamin D status of the winter pastures might fully account for outbreaks of ovine rickets in the field. Accordingly vitamin D assays were carried out on a number of different types of pasture sampled at different seasons of the year. Table 1 summarises the results of this work, which indicates that the vitamin D content of pasture does not depend upon the season of the year in which it is grown.

(2) *A Rachitogenic Effect from Dosing a Permanent-pasture Extract*

When one sample taken from a permanent pasture in autumn was assayed for vitamin D, the average bone ash response to a daily dose of the extract though significantly higher than the level of the control group, was smaller than the response to the lowest vitamin D standard (0.05 I.U. vitamin D daily) employed in the assay (Table 2). The assay was therefore repeated, but in this case 0.1 I.U. vitamin D was given with each daily dose of the pasture extract to ensure that the response obtained would lie upon that part of the log dose/response line known to be linear. The average percentage bone ash resulting from this dose was thus expected to lie between those obtained from standards of 0.1 and 0.2 I.U. vitamin D and to be greater than the response to 0.1 I.U. vitamin D daily by an amount contributed by the vitamin D of the pasture extract. The results (Table 3) indicated the presence of a bone-ash-depressing factor, since the response was significantly less than expected from the amount of vitamin D added. Only the relevant data from these assays are presented in Tables 2 and 3.

TABLE 2. Slight Antirachitic Potency of a Permanent-pasture Extract

Group	Daily Dose per Rat	Av. % Femur Ash	S. group 2
1	—	27.26	—2.62**
2	Extract from 0.15 g. pasture	29.88	
3	0.05 I.U. vitamin D	31.97	2.09*

S.E. for group means ± 0.64 (12 rats per group)

Throughout the Tables presented in this paper:
S. denotes difference from the mean for reference group.
* difference significant at 5% level.
** difference significant at 1% level.

TABLE 3. The Rachitogenic Potency of the Permanent-pasture Extract

Group	Daily Dose per Rat	Av. % Femur Ash	S. Group 2
1	0.10 I.U. vitamin D	37.20	3.42**
2	Extract from 0.15 g. pasture plus 0.10 I.U. vitamin D	33.78	
3	—	31.50	—2.28*

S.E. for group means ± 0.62 (12 rats per group)

(3) *The Antirachitic Properties of an Extract of Dead Brown Leaves and the Rachitogenic Effect of the Green-leaf Extract*

From the results shown in Table 1 it was apparent that the vitamin D content of pasture bore no relation to the season of the year in which the sample was taken, but it was noted that those samples in which appreciable amounts of the vitamin were present contained considerably larger amounts of dead brown leaves. It seemed possible therefore that the vitamin D of a pasture was present mainly in the dead leaves, while the green growing leaves contained practically none. A permanent-pasture sample cut in early autumn was separated into green leaves and dead brown leaves, a fraction consisting of leaves containing both green and brown damaged tissue being discarded. The extract from each fraction was assayed for vitamin D. The results indicated that vitamin D was present to the extent of approximately 100 I.U./100g. D.M. in the dead leaf fraction (Table 4).

TABLE 4. VITAMIN D IN DEAD BROWN LEAVES

Group	Daily Dose per Rat	Av. % Femur Ash
1	—	31.88
2	0.10 I.U. vitamin D	35.58
3	0.20 I.U. vitamin D	38.23
4	Extract from 0.17 g. dried brown leaves	37.67

S.E. for group means ± 0.69 (12 rats per group)

The average percentage femur ash of rats dosed with the extract from the green leaves was significantly lower than that of a control group which received no vitamin D supplement (Table 5). Apparently some rachitogenic substance was present in the extract.

TABLE 5. A RACHITOGENIC FACTOR IN GREEN LEAVES

Group	Daily Dose per Rat	Av. % Femur Ash	S. Group 1
1	—	32.91	
2	Extract from 0.17 g. dried green leaves	28.08	—4.83**

S.E. for group means ± 0.71 (12 rats per group)

(4) *The Rachitogenic Effect of a Green-leaf Extract on Rats Receiving Varying Amounts of Vitamin D*

An extract of Italian ryegrass (green leaves only) was dosed to rats as indicated in Table 6. The results of this trial suggested that a dose

of rachitogenic factor, which significantly depressed the bone ash response to doses of vitamin D, may have less effect on calcification in the case of animals receiving no vitamin D.

TABLE 6. A RACHITOGENIC FACTOR IN GREEN LEAVES OF ITALIAN RYEGRASS

I.U. Vit. D. Dosed Daily per Head	Av. % femur ash		
	No Green-leaf Extract Given	Extract Equivalent to 0.15 g. Dried Leaves Dosed Daily per Head	Difference Due to Leaf Extract
0.0	29.88	29.13	—0.75
0.2	38.13	33.69	—4.44**

S.E. for group means ± 0.89 (12 rats per group)

TABLE 7. THE RACHITOGENIC EFFECT OF GREEN-LEAF EXTRACTS ON RATS RECEIVING A SUPPLEMENT OF VITAMIN D

Experiment	No. Rats per Group	Daily Dose per Rat		Av. % Femur Ash		
		I.U. Vit. D	Dried Leaves Dosed as $CHCl_3$ Extract			S. Control group
1	12	0.10 0.10	— 0.15g. Permenant pasture	37.20 33.78	±0.62	—3.42**
2	12	0.10 0.10	— 0.15g. Italian ryegrass	33.68 30.89	±0.89	—2.79*
3	10	0.20 0.20	— 0.15g. Oats	34.90 30.53	±0.54	—4.37**
4	12	0.20 0.20	— 0.15g. Oats	36.67 32.60	±0.85	—4.07**
5	12	0.20 0.20	— 0.15g. Oats	37.38 34.36	±0.88	—3.02*
6	6	0.20 0.20	— 0.15g. Permanent pasture	39.85 32.57	±1.71	—7.28*
7	6	0.20 0.20	— 0.15g. Perennial ryegrass	36.32 31.43	±1.24	—4.89*
8	6	0.20 0.20	— 0.15g. Oats	36.97 31.78	±1.07	—5.19**
9	12	0.20 0.20	— 0.15g. Italian ryegrass	38.13 33.69	±0.89	—4.44**
10	7	0.30 0.30	— 0.15g. Oats	34.20 27.31	±1.36	—6.89**
11	12	0.30 0.30	— 0.15g. Italian ryegrass	37.97 35.41	±0.59	—2.56**

(5) *The More-pronounced Effect of the Rachitogenic Factor in Rats Receiving a Vitamin D Supplement*

The results of a number of tests for the presence of a rachitogenic factor in different samples of greenfeeds are summarized in Tables 7 and 8. While a marked rachitogenic effect was evident in those trials in which extracts of green leaves from a number of pasture plants were given to rats receiving some vitamin D (Table 7), the effect was not always appreciable when the test animals were given no vitamin D supplement (Table 8).

TABLE 8. THE RACHITOGENIC EFFECT OF GREEN-LEAF EXTRACTS ON RATS RECEIVING NO VITAMIN D SUPPLEMENT

Experiment	No. Rats per Group	Daily Dose per Rat (Wt. Dried Leaves Dosed as $CHCl_3$ Extract)	Av. % Femur Ash	S.	control group
1	12	— 0.3 g. Oats	26.43 26.25	±0.63	—0.18
2	10	— 0.3 g. Oats	27.89 26.77	±0.93	—1.12
3	12	— 0.15 g. Oats	28.36 28.43	±0.52	+0.07
4	11	— 0.15 g. Perennial ryegrass	28.62 27.71	±0.75	—0.91
5	11	— 0.15 g. Oats	29.54 26.22	±0.97	-3.32*
6	11	— 0.15 g. Italian ryegrass	29.88 29.13	±0.89	—0.75
7	9	— 0.15 g. Oats	30.20 29.22	±1.11	—0.98
8	12	— 0.15 g. Oats	31.22 27.76	±0.79	—3.46*
9	11	— 0.15 g. Oats	32.65 29.72	±0.76	—2.93*
10	11	— 0.15 g. Oats	32.83 27.79	±0.74	5.04**
11	12	— 0.17 g. Permanent pasture	32.91 28.08	±0.71	—4.83**

The data in Table 8, presented in the order of increasing percentage bone ash of the control groups, made it appear likely that the rachitogenic effect was less pronounced in those cases in which the degree of rickets, produced by the basal ration, was more severe. The possibility was considered that the rachitogenic factor may interfere directly with the function of vitamin D within the body, and if this were so, it would be expected that its rachitogenic effect would be evident to a more marked extent in animals receiving appreciable amounts of vitamin D in their diet, or in those in which the initial body store of the vitamin was relatively large. It seems unlikely that either the olive oil employed as a dosing vehicle or the McCollum 3143 ration would be entirely deficient

in vitamin D, and, though small, the amounts may vary considerably in different samples of oil or in the ration made up of constituents from different sources. These considerations may largely account for the apparent variation in the rachitogenic potency of an extract when no vitamin D supplement is given.

(6) *The Effect of the Rachitogenic Factor Dosed Orally on the Bone-ash Response to Vitamin D Given Subcutaneously*

The possibility of the green-leaf extract containing some factor that interfered with absorption of vitamin D from the gut seemed unlikely from the results of a trial in which the extract was administered to the test animals orally and vitamin D subcutaneously (Table 9).

TABLE 9. EFFECT OF THE RACHITOGENIC FACTOR DOSED *per os* ON RATS RECEIVING A VITAMIN D SUPPLEMENT SUBCUTANEOUSLY

Group	Daily Oral Dose per Rat	Weekly Subcutaneous Dose per Rat	Av. % Femur Ash	S. group 1
1	—	2.0 I.U. vitamin D	34.20	
2	Extract from 0.15 g. green oats	2.0 I.U. vitamin D	27.31	—6.89**

S.E. for group means ± 1.36 (7 rats per group)

(7) *An Attempt to Concentrate the Rachitogenic Factor*

In a preliminary fractionation trial, in which concentration of the rachitogenic factor was attempted, 35 g. of the chloroform extract from green oat leaves was extracted six times with 100 ml. volumes of absolute alcohol, by vigorously shaking the mixture in each case for 24 hours. Thus was obtained 21.5 g. of an alcohol-soluble fraction, F_1, and 13.5 g. of an insoluble residue, F_2. The results of a test for the presence of the rachitogenic factor in these two fractions showed that both contained an appreciable quantity (Table 10). However, no assessment could be made as to the relative amounts of the factor present in such fractions, so that, before any further attempt to concentrate the factor was made, it seemed desirable to examine more closely its rachitogenic properties in the hope that some quantitative relationship between dose and bone-ash-depressing effect might lead to a method whereby the factor could be assayed or its relative concentration in solutions could be determined.

(8) *The Effect of a Fixed Dose of the Rachitogenic Factor on Bone-ash Responses to Varying Vitamin D Supplements*

The way in which a fixed dose of the rachitogenic factor, dosed as the chloroform extract of green oats containing practically no dead brown tissue, affects the "normal"* bone ash response to increasing amounts of vitamin D is indicated by the results of a trial depicted in Fig. 1.

* The "normal" bone-ash response referred to on this and on subsequent pages refers to the response to vitamin D of rats on the McCollum ration receiving no supplement of the rachitogenic factor.

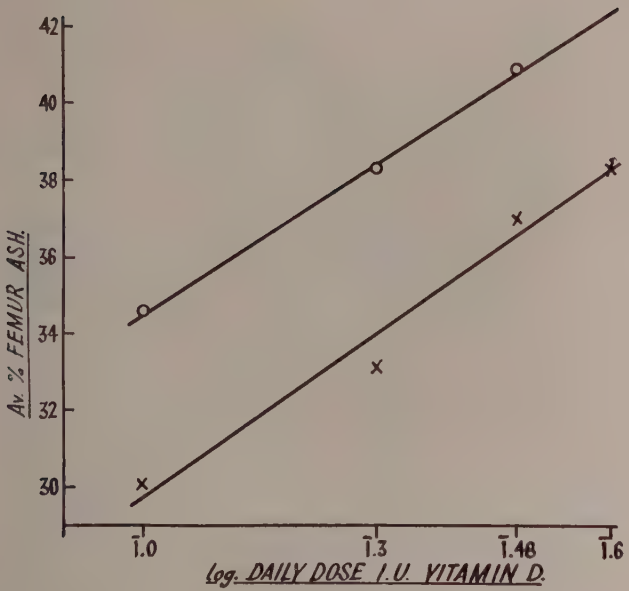

Fig 1.—Effect of a fixed dose of green-leaf extract on bone-ash response to vitamin D.
O Response to vitamin D with no green-leaf extract.
X Response to vitamin D plus fixed daily dose of green-leaf extract equivalent to 0.15 g. dry matter per head.
Each group contained 7 rats.

TABLE 10. INCOMPLETE EXTRACTION OF THE RACHITOGENIC FACTOR WITH ALCOHOL FROM THE CHLOROFORM-SOLUBLE FRACTION OF GREEN OATS

Group	Daily Dose per Rat		Av. % Femur Ash	S. group 1
	I.U. Vitamin D	Fraction Dosed to Test Animals		
1	0.20	—	34.47	—5.07**
2	0.20	02 g. F1†	29.40	—5.99**
3	0.20	02 g. F2	28.48	
S.E. for group means ± 1.02 (6 rats per group)				

†F1 = alcohol-soluble material
F2 = alcohol-insoluble material

Statistical analysis of the results indicated an equally good linear relationship between percentage ash and log. dose for both series. The two regression coefficients were not significantly different but the depressed responses of the extract-dosed groups were such that vitamin D potency was approximately halved. The difference between means of groups receiving the same vitamin D supplement was in each case significant at the 1% level.

(9) *The Depressing Effect of Increasing Doses of the Factor on Bone-ash Response to a Fixed Dose of Vitamin D*

In order to study the effect of increasing doses of the rachitogenic factor on the bone-ash response to a fixed dose of vitamin D, groups of rats, each receiving daily 0.15 I.U. vitamin D per head, were dosed with increasing amounts of a chloroform extract of green pasture leaves. The results of this trial are shown in Fig. 2.

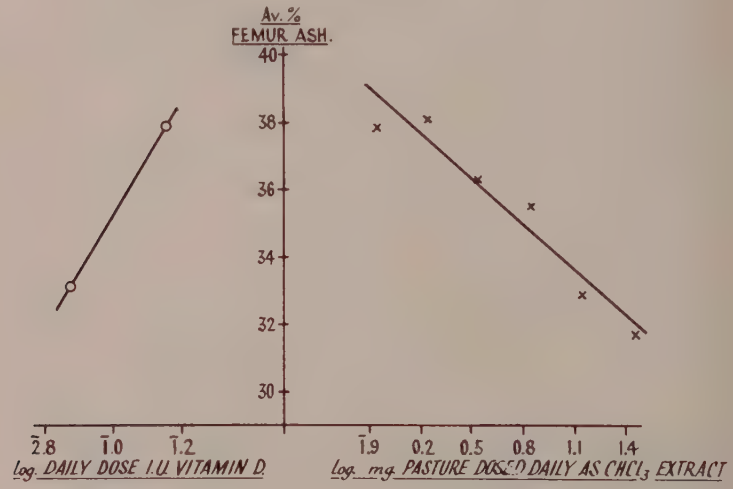

Fig. 2.—Effect of increasing doses of pasture extract on bone-ash response to a fixed daily dose of vitamin D.
O "Normal" bone-ash response to vitamin D.
X Bone-ash response to a daily dose of 0.15 I.U. vitamin D plus green-leaf extract.
Each group contained 18 rats.

Comparable results were obtained in eight other similar trials in which was determined the depressing effect of increasing doses of extracts of green leaves from other samples of oats and perennial ryegrass given to rats receiving the same daily supplement of vitamin D. Statistical analysis in all but one of these nine trials showed that linear regression of mean percentage ash on log. dose rachitogenic factor was significant (at the 1% level in 6 trials and at the 5% level in two trials). The regression coefficients varied between the limits of —2.23 and —4.50 and there was no significant difference between any of the established values.

Since it was shown (Tables 7 and 8) that the effect of the rachitogenic factor was much more pronounced in rats receiving a vitamin D supplement than in those that were given none, and since field trials (Fitch, 1943; Fitch and Ewer, 1944) in the case of sheep had indicated that supplements rich in calcium and/or phosphorus did not reduce the severity of rickets produced on green feeds, it was assumed that the factor directly interfered with vitamin D function. It was therefore expected that a fixed dose of the factor would have a constant depressing effect upon bone-ash response to a vitamin D supplement and that determination of the extent to which the vitamin D calcifying

effect was depressed would provide a measure of the rachitogenic factor intake. An assay method devised and briefly outlined elsewhere (Grant, 1953) was subsequently shown to be inapplicable although when employed to determine the rachitogenic-factor content of green oats at different stages of growth results were obtained that agreed remarkably well with later and more precise determinations.

Since the regression of mean percentage ash on log. dose rachitogenic factor was linear in the case of rats receiving a fixed vitamin D supplement, it was evident that an assessment of relative concentrations of the factor in materials containing no vitamin D could be made by directly comparing the extent to which they would depress bone-ash response to vitamin D in litter-mate controlled experiments. The presence of vitamin D in an extract of green leaves in amounts that could markedly affect such tests was considered unlikely, as no evidence for the presence of the vitamin in the green leaf has been found, and the results of trials have indicated that the antirachitic effect of appreciable amounts would not be completely masked by the factor. The procedure of measuring relative concentrations was adopted in most of the subsequent work involved in concentrating and isolating the active principle. Because of the low gradient in the slope of the log. dose/response line it was apparent that the test would be subject to large error, but it was considered that it would be sufficiently accurate to enable the fate of the factor to be followed throughout any of the processes involved in attempts at its isolation.

(10) *Chromatographic Fractionation of Green-feed Extracts*

In preliminary experiments, chloroform extracts of green leaves were passed through activated alumina and the columns subsequently developed with chloroform. None of the bands of the chromatograms exhibited a rachitogenic effect, which was invariably confined to the fraction most easily eluted and not readily resolved with chloroform as the solvent. Since this solvent exhibits an appreciable degree of polarity, which causes rapid development of the chromatogram and movement of less strongly adsorbed substances through the column at such a rate that resolution into separate bands is slow, it was obviously not suitable for the fractionation of such a readily eluted substance as the rachitogenic factor appeared to be. The possibility of using petroleum ether as a solvent was therefore investigated, and it was found that cold petroleum ether (b.p. 30-60°C.) extracted most of the rachitogenic factor from the dry powdered greenfeed (Table 11).

Initially, some difficulty was experienced in obtaining an even band formation when attempts were made to chromatograph the petroleum-ether extract on an alumina column. It appeared that some or one of the extract constituents was precipitated in the upper bands, resulting at first in uneven formation of the lower bands and then in a gradual decrease in the rate at which the solution moved through the column, until finally the column became completely clogged. Disturbing the upper bands with a stirring rod caused only a temporary resumption of liquid flow. This difficulty was overcome by the addition to the petroleum-ether extract of 2.5% chloroform which was apparently sufficient to keep the offending material in solution without unduly increasing the polarity of the solvent. It was later discovered that this clogging effect was related to the quality of the dried leaves from which

TABLE 11. SUITABILITY OF PETROLEUM ETHER FOR EXTRACTING THE RACHITOGENIC FACTOR FROM DRIED AND POWDERED GREEN LEAVES

Group	Daily Dose per Rat		Av. % Femur Ash	
	I.U. Vitamin D	Equivalent Wt. Dry Pasture (mg) Dosed as Extract		S. group 1
1	0.2	—	34.40	
2	0.2	10†	33.03	—1.37
3	0.2	30†	31.37	—3.03*
4	0.2	10‡	34.33	—0.07
5	0.2	30‡	35.15	+0.75

S.E. for group means ± 0.91 (6 rats per group)

† Petroleum-ether extract of dried oats.

‡ Chloroform extract of residue insoluble in petroleum ether

the extract was prepared. The effect was almost entirely absent in the case of petroleum-ether extracts from grass freshly dried at low temperature, but with extracts from samples dried at 110°C., or stored for a considerable period before extraction, the effect was appreciable.

A marked difference in the appearance of chromatograms from extracts of greenfeeds dried at different temperatures or stored for different lengths of time was also noted. Probably owing to decomposition of the leaf constituents, the number of bands on the chromatogram prepared from overheated or long-stored pasture was appreciably greater.

(11) *Detection of the Rachitogenic Factor in the Carotene Band of the Chromatogram*

Tests with columns prepared from petroleum-ether extracts and developed with the same solvent also pointed to the presence of the active principle in the lower bands. Tables 12 and 13 summarize the description of a typical alumina chromatogram obtained from the petroleum-ether extract of dried green oats, the fractions into which the column was mechanically divided, and the result of a potency test on each fraction after elution and dosing in olive oil to the test animals. The column was 1 cm. in diameter and approximately 26 cm. in height. The amount of material fractionated on the column was the petroleum-ether extract (100 ml.) from 60 g. of dried oats. A further 25 ml. of petroleum ether was added after the extract had passed into the column. Previous work had indicated the presence of the factor either in the first eluted zone (colourless band) or in the following band coloured by the presence of carotene. It was found that the addition of a little azobenzene to the original petroleum-ether extract conveniently split the colourless zone into three distinct bands as indicated in Table 12.

TABLE 12. CHROMATOGRAPHIC FRACTIONATION OF PETROLEUM ETHER EXTRACT OF GREEN OATS ON AN ALUMINA COLUMN

Band on Chromatogram			Fractions Dosed to Test Animals
No.	Width (cm.)	Colour	
1	3.5	Light green	a
2	0.2	Dark green	
3	1.5	Grey turning to black	
4	0.5	Light yellow	
5	0.1	Red	b
6	0.2	Yellow	
7	0.1	Red	
8	6.0	Buff	c
9	0.2	Orange	
10	7.0	Red (carotene band)	d
11	7.0	Colourless	e
12	Eluted	Yellow (azobenzene band)	f
13	Eluted	Colourless	g

Table divided at points at which column mechanically divided

The eluate from each fraction, after removal of the solvent, was taken up in olive oil and the rachitogenic potency tested as shown in Table 13.

A significant bone-ash-depressing effect was evident in the case of group 5 (fraction d) only.

These results not only confirmed previous observations that the factor was present in the lower bands of the chromatogram but pointed to the "carotene band" as the one in which the factor predominated. The possibility suggested by the results that each zone on either side of the carotene band might contain a small amount of the factor, could be accounted for by the fact that no sharp mechanical division of the zones is possible and that in the cutting of the column the contents of each band is contaminated by constituents of the bands immediately above and below it. Although at this time the zone containing the rachitogenic factor was referred to as the "carotene band" it was not suspected that carotene itself, as was later discovered, was the factor being investigated. Apart from carotene the band appeared to be made up of many other

TABLE 13. TEST OF FRACTIONS FROM CHROMATOGRAM FOR RACHITOGENIC POTENCY

Group	Daily Dose per Rat		Av. % Femur Ash	S. group 1
	I.U. vitamin D	Fraction from Chromatogram Equivalent to 100 mg. Dried Oats		
1	0.15	—	39.58	
2	0.15	a	38.86	—0.72
3	0.15	b	40.11	+0.53
4	0.15	c	37.58	—2.00
5	0.15	d	34.29	—5.29**
6	0.15	e	38.33	—1.25
7	0.15	f	39.31	—0.27
8	0.15	g	39.82	+0.24

S.E. for group means ± 0.90 (12 rats per group)

constituents, and it was only the fact that the red colour of carotene conveniently marked its position in the column that it was so named.

Before an attempt was made to isolate the active principle it was considered desirable to determine the stage of plant growth at which the factor would be present in greatest concentration and to determine its stability under different conditions, particularly in contact with absorbents, in the hope that chromatography would provide the most simple and rapid method of concentration.

(12) *Variation of Rachitogenic-factor Content with Stage of Plant Growth*

The results presented in Table 14 indicated that the concentration of the rachitogenic factor in a crop of green oats, determined by the proposed assay method, was highest during the earlier stages of plant growth. This crop was grown under excellent conditions and growth rate was rapid.

(13) *The Stability of the Rachitogenic Factor when Greenfeed was Dried by Different Methods*

To determine the effect of different drying methods on the rachitogenic-factor content of greenfeed, a sample of permanent pasture consisting of green leaves, mainly of perennial ryegrass, was thoroughly mixed and divided into three equal lots. Lot 1 was dried in a hot-air oven at 105°c. for four hours. Lot 2 was dried by exposure to wind and direct sunlight for seven hours. Lot 3 was dried in shade by fan at room temperature for 3 days.

The extract from each lot was dosed at two levels to the test animals (Fig. 3). At each dosage level there was no significant difference

TABLE 14. VARIATION OF RACHITOGENIC-FACTOR CONTENT OF GREEN OAT LEAVES WITH STAGES OF PLANT GROWTH

Assay	Stage at which Oat Crop Sampled: No. of Days Growth	Description of Crop	Rachitogenic-factor content. Arbitrary Units/g. D.M.
1	4	Young shoots 1-3 in. high	200
2	14	5-6 in. high	320
3	23	7-9 in. high	320
4	35	9-12 in. high. Considerable stalk development	100
5	50	Crop coming into flower	50

between the bone-ash-depressing effect of any of the three extracts and it was therefore assumed that the factor was not destroyed to any great extent when pasture was dried by any of these methods. The rachitogenic effect of the larger dose for each lot was highly significant.

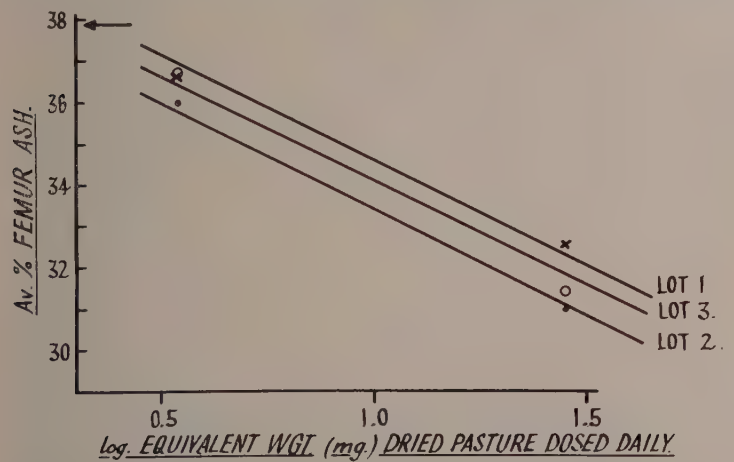

Fig. 3.—The Effect of different drying methods on the rachitogenic-factor content of green feed.

◄ Normal response to 0.15 I. U. vitamin D daily.
X Response depressed by extract of lot 1, dried at 105°c.
• Response depressed by extract of lot 2, dried by wind in direct sunlight.
O Response depressed by extract of lot 3, dried at room temperature in shade.
Each group contained 6 rats.

(14) *The Apparent Stability of the Rachitogenic Factor in Contact with Activated Alumina*

In the event of the readily available alumina having a destructive action on the factor, a test was simultaneously conducted with a silica

column. The petroleum-ether extract from 100 g. dried green oats was divided into three equal volumes. The rachitogenic potency of the first untreated fraction was directly compared with those of the other two solutions treated as follows:

The second fraction was passed through a column of alumina 8 cm. high and 1 cm. in diameter, and the eluate up to and including the zone above the "carotene" band was collected.

The third fraction was passed through a silica column of the same size and the same bands eluted.

The solvent was removed from each of the three solutions and the residue dissolved in olive oil and dosed in equivalent amounts to the test animals. The results which appear in Table 15 indicated that the rachitogenic factor was not destroyed to any great extent by contact with these adsorbents. There was no significant difference between any of the means of groups 2, 3, and 4.

TABLE 15. A TEST FOR THE STABILITY OF THE RACHITOGENIC FACTOR IN CONTACT WITH ACTIVATED ALUMINA OR SILICA

Group	Daily Dose per Rat		Av. % Femur Ash	S. Group 1
	I.U. Vitamin D	Equivalent Wt. Dry Oats dosed		
1	0.15	0	36.68	
2	0.15	0.13 g. as untreated extract	32.32	—4.36**
3	0.15	0.13 g. as extract after contact with activated alumina	31.74	—4.94**
4	0.15	0.13 g. as extract after contact with activated silica	31.17	—5.51**

S.E. for group means ± 0.98 (9 rats per group)

Although the results were not statistically significant similar tests suggested (1) that destruction of the rachitogenic factor took place to some extent when the dried powdered greenfeed was stored for three months at room temperature so that, as a precautionary measure in subsequent work, material containing the factor was as far as possible kept in the refrigerator at —20°C.

(2) that green oats growing slowly during a dry spell in early autumn contained a smaller amount of the factor than a rapidly grown crop cut after the same period of growth.

(15) *Isolation of Carotene and Demonstration of its Rachitogenic Effect*

Having ascertained the rate and stage of growth of the green oat plant at which the rachitogenic factor would probably be present in high concentration, and after showing that the rachitogenic factor

was comparatively stable under all the conditions to which it would be subjected during the proposed extraction and concentration process, an attempt was made on a moderately large scale to isolate the active principle.

A crop of green oats grown rapidly in mid autumn was cut when it was approximately six inches high. The green leaves were scattered not too thickly on horizontal wire-netting racks constructed in the field two feet above ground level and, as the cutting was left till the morning of a dry windy day, the material when collected in the late afternoon was fairly well dried out, sufficiently so as to be rapidly dried at 35–40°c. in a small grass-drying machine. The resulting material was finely powdered in a hammer-mill. Ten kg. of the powder were extracted by firmly pressing into an extraction tower and allowing petroleum ether (b.p. 40-60°c.) to percolate through the mass. After some eight litres of the solvent had passed through, the extraction, judging from the colour of the solution dripping out, was almost complete. The solution was chromatographed with a battery of 25 columns of alumina, each 3 cm. in diameter, and 10 cm. in height. In each case the material collected from the eluate contained all the constituents of the red "carotene-band" and a little of those of the bands immediately above and below. The combined solutions were evaporated in vacuo at 35-40°c. to approximately 120 ml. of a dark-red liquid. Removal of the solvent from a small test sample indicated that it contained 61.0 g. of non volatile material. The solution was left at —20°c. for three weeks to allow as much as possible of the insoluble material to crystallize out. It was then filtered at —20°c. and the residue washed with pure petroleum ether cooled to the same temperature.

Weight of crystalline solid was 12.1 g. (Fraction A). Therefore, weight of material remaining in solution was 48.9 g. (Fraction B).

A determination of the relative rachitogenic potency of these fractions indicated that A contained 6-7 times the concentration of the factor remaining in B (Fig. 4).

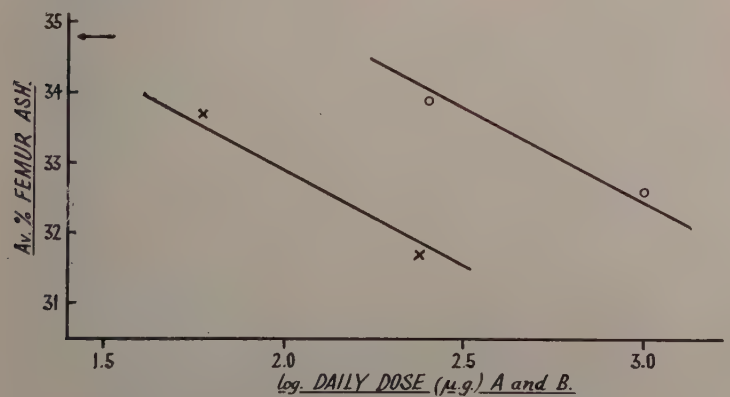

Fig. 4.—The relative rachitogenic potency of fractions A and B.
◀ Normal response to daily dose of 0.15 I.U. vitamin D.
X Response to 0.15 I.U. vitamin D + A.
O Response to 0.15 I.U. vitamin D + B.
Each group contained 12 rats.

The possibility that carotene might be the rachitogenic factor was first considered at this stage, for, judging from the colour of the two fractions, both contained a considerable amount of that substance.

Carotene was separated and purified from fraction A as follows. The solubility of all the constituents of fraction A in chloroform was found to be high. It was completely dissolved in a small volume of this heated solvent (approximately 20 ml.), and to this was added 300 ml. of petroleum ether (b.p. 40-60°c.) heated to near boiling point. The solution was left to cool at room temperature. Dark-red crystals of carotene rapidly separated from solution and these were filtered off as soon as an appreciable quantity had separated, and before any of the other constituents of fraction A started to crystallize. The carotene thus prepared was further purified by again dissolving in a small volume of chloroform and crystallizing out with petroleum ether. The weight of carotene thus isolated was 0.47 g. and the rachitogenic properties of this product can be seen from the results of the trial shown in Fig. 5.

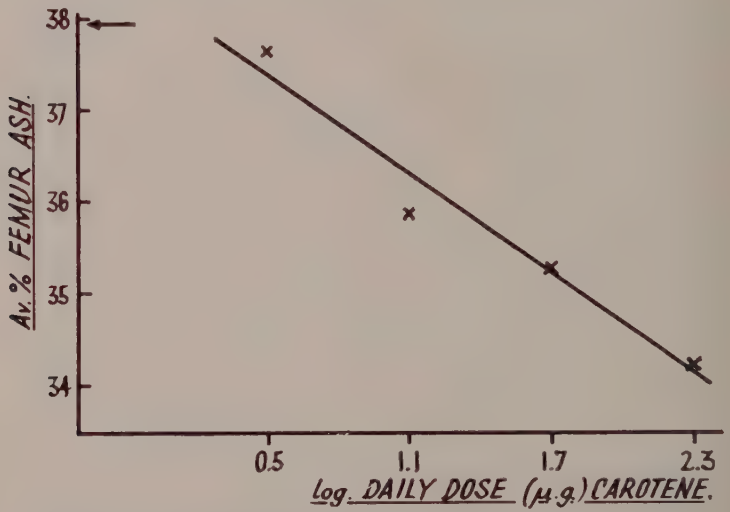

Fig. 5.—The rachitogenic effect of carotene separated from fraction A.
← Normal response to 0.15 I.U. vitamin D daily.
X Response to 0.15 I.U. vitamin D daily + carotene.
Each group contained 12 rats.

(16) *The Rachitogenic Effect of a Commercial Preparation of Carotene*

The results of the previous trial were confirmed by dosing increasing amounts of a commercial preparation of carotene (B.D.H.: 90%β–10%α) to rats on the McCollum ration receiving a supplement of vitamin D (Fig. 6).

(17) *The Rachitogenic Properties of Vitamin A (Synthetic)*

The results of a similar trial in which carotene was replaced by pure crystalline vitamin A acetate are shown in Table 16.

TABLE 16. RACHITOGENIC PROPERTIES OF PURE VITAMIN A ACETATE

Group	Daily Dose per Rat		Av. Wt. Dry Femurs		Av. % Femur Ash	
	I.U. Vit. D	I.U. Vit. A	(g. × 10^4)	S. group 8		S. group 2
1	0.075	0	1500	207**	33.15	
2	0.15	0	1530	237**	35.09	
3	0.15	5	1488	195**	33.07	—2.02*
4	0.15	20	1457	164*	32.34	—2.75**
5	0.15	80	1440	147*	31.43	—3.66**
6	0.15	320	1484	191**	30.20	—4.89**
7	0.15	1280	1460	167**	28.37	—6.72**
8	0.15	5120	1293		29.73	—5.36**
	S.E. for group means (24 rats per group)		± 44		± 0.70	

Analysis of Variance (Percentage ash groups 3–7)

Nature of Variation	d.f.	m.s.
Linear regression	1	319.23**
Deviations	3	3.76
Between doses	4	82.63**
Between litters	23	16.49*
Residual	92	9.39

Regression co-efficient = —1.916 ± 0.328

The high vitamin A dose administered to the animals of group 8 had a toxic effect. These rats were relatively unthrifty and the average weight of the dry fat-free femur was significantly lower than that of the other groups, which did not differ to any appreciable extent. Percentage-ash values from group 8 were therefore not included in the analysis of variance of results from the series of vitamin A supplemented groups. The results were similar to those reported in section 9 in which the depressing effect of green-leaf extracts on bone-ash response to vitamin D was investigated. The regression coefficient in this case (—1.916) was outside the range observed (—2.23 to —4.50) in the previous trials.

(18) *Comparison of Rachitogenic Potency of Equivalent Amounts of Carotene and Vitamin A*

The similar rachitogenic potency of equivalent amounts of carotene and vitamin A was demonstrated in a direct litter-mate comparison trial,

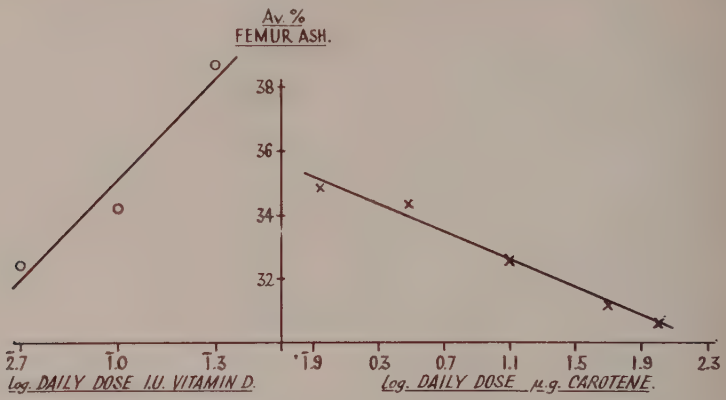

Fig. 6.—The rachitogenic effect of carotene (B.D.H. 10%α, 90%β).
O Normal bone ash response to vitamin D.
X Response to 0.15 I.U. vitamin D + carotene.
Each group contained 24 rats.

in which it was also shown that the total rachitogenic potency of a chloroform extract of dried green oats could be accounted for by its carotene content (Table 17). In this experiment the carotene content of the oats was estimated by the method of McGillivray (1952). The carotene used was a mixture of 90%β and 10%α, and was considered to be approximately equivalent to 50 I.U. vitamin A. There was no significant difference between the rachitogenic potency of any of the three preparations.

TABLE 17. THE TOTAL RACHITOGENICITY OF GREEN OATS EXTRACT ACCOUNTED FOR BY ITS CAROTENE CONTENT AND THE SIMILAR RACHITOGENIC POTENCY OF EQUIVALENT AMOUNTS OF CAROTENE AND VITAMIN A

Group	Daily Dose per Rat		Av. % Femur Ash	S. group 1
	I.U. Vitamin D	Rachitogenic Substance		
1	0.15	0	35.28	
2	0.15	Oats extract containing 33 μg carotene	30.80	—4.48**
3	0.15	33 μg carotene	30.93	—4.35**
4	0.15	50 I.U. vitamin A	30.00	—5.28**

S.E. for group means ± 0.81 (24 rats per group)

Having demonstrated the rachitogenic properties of carotene and vitamin A, it seemed desirable to examine more thoroughly the way in which they affected the normal bone-ash response to vitamin D.

(19) *The Rachitogenic Effect of β-carotene and/or Vitamin A in Rats receiving No Vitamin D or Relatively Small Supplements*

Preliminary trials, before the identity of the rachitogenic factor was known, indicated that when no vitamin D supplement was given to the test animals the percentage bone ash induced by the basal McCollum 3143 rachitogenic ration was not greatly depressed by the factor, whereas an appreciable response to vitamin D was markedly reduced. This observation has been confirmed by the results of trials in which increasing amounts of β-carotene were dosed to groups of rats receiving the basal ration with no vitamin D supplement (Table 18), and by administering small but increasing doses of vitamin D to groups receiving a large fixed dose of vitamin A (Table 19).

TABLE 18. THE EFFECT OF β-CAROTENE ON BONE CALCIFICATION IN RATS RECEIVING NO VITAMIN D SUPPLEMENT

Group	Daily Dose per Rat μg β-carotene	Av. % Femur Ash	S. group 1
1	0	26.36	
2	0.781	26.52	+0.16
3	3.125	25.62	—0.74
4	12.5	25.63	—0.73
5	50.0	25.54	—0.82
6	200.0	27.75	+1.39

S.E. for group means ± 0.60 (24 rats per group)

TABLE 19. EFFECT OF VITAMIN A ON BONE ASH RESPONSES TO SMALL TO INTERMEDIATE VITAMIN D SUPPLEMENTS

I.U. Vit. D Dosed Daily per Head	Av. % Femur Ash		
	No Vit. A Supplement	100 I.U. Vit. A Dosed Daily per Head	Difference Due to Vit. A Supplement
0.025	28.00	—	—
0.05	28.48	28.05	—0.43
0.10	31.58	27.40	—4.18**
0.20	36.50	31.84	—4.66**
0.40	—	36.20	

S.E. for group means ± 0.82 (24 rats per group)

(20) *The Rachitogenic Effect of Vitamin A on Bone-ash Response to Intermediate and Adequate Vitamin D Supplements*

The effect of vitamin A on the bone-ash response to intermediate and what can be regarded as adequate vitamin D intakes can be seen from the results obtained in the following trials. It seems certain that over most of its linear range, the slope of the log vitamin D dose/response regression line is not appreciably affected by a fixed dose of vitamin A (Tables 20 and 21) and that as the vitamin D intake approaches the daily requirement of the animal, so does the rachitogenic effect of vitamin A decrease (Table 22).

The results of these experiments (19 and 20) are consistent with the relationships indicated in the hypothetical Fig. 7.

TABLE 20. EFFECT OF A DAILY DOSE OF VITAMIN A (100 I.U. PER HEAD) ON RESPONSE TO INTERMEDIATE SUPPLEMENTS OF VITAMIN D

I.U. Vit. D Dosed Daily per Head	Av. % Femur Ash		
	No Vit. A Supplement	100 I.U. Vit. A Dosed Daily per Head	Difference Due to Vit. A Supplement
0.1	29.10	—	
0.2	32.57	30.64	—1.93*
0.4	36.40	34.24	—2.16**
0.8	39.55	37.59	—1.96*

S.E. for group means ± 0.53 (24 rats per group)

Analysis of Variance

Nature of Variation	Unsupplemented Groups		Vitamin A Supplemented Groups	
	d.f.	m.s.	d.f.	m.s.
Linear regression	1	1483.69**	1	578.87*
Deviations	2	0.94	1	0.31
Between doses	3	495.19**	2	259.59**
Between litters	23	33.07	23	10.13
Residual	69	7.05	46	6.61
Regression coefficient	11.680 ± 0.805		11.535 ± 1.232	

TABLE 21. EFFECT OF A DAILY DOSE OF VITAMIN A (1000 I.U. PER HEAD) ON RESPONSE TO INTERMEDIATE SUPPLEMENTS OF VITAMIN D

I.U. Vit. D Dosed Daily per Head	Av. % Femur Ash		
	No Vit. A Supplement	1000 I.U. A Dosed Daily per Head	Difference Due to Vit. A Supplement
0.1	29.03	—	—
0.2	32.32	28.04	—4.28**
0.4	35.95	31.75	—4.20**
0.8	39.27	34.76	—4.51**

S.E. for group means ± 0.60 (24 rats per group)

Analysis of Variance

Nature of Variation	Unsupplemented Groups		Vitamin A Supplemented Groups	
	d.f.	m.s.	d.f.	m.s.
Linear regression	1	1416.25**	1	542.04*
Deviations	2	0.26	1	1.98
Between doses	3	472.25**	2	272.01**
Between litters	23	25.41	23	10.23
Residual	69	8.87	46	8.34
Regression coefficient	11.412 ± 0.903		11.162 ± 1.385	

(21) *The Effect of Initial Vitamin A Liver Storage on the Bone-ash Response to Vitamin D*

The probability that an appreciable difference in the initial level of vitamin A storage in the test animals could affect the bone-ash response to vitamin D was indicated by the results of a trial (Table 23) in which, during the pre-weaning stage, half of the test animals were each given five daily doses of 2,000 I.U. vitamin A. All the animals were then weaned on to the McCollum 3143 rachitogenic ration and each dosed daily with 0.3 I.U. vitamin D throughout the 28-days trial period.

(22) *The Possibility of Vitamin A Affecting Phosphorus Utilization Rather than Directly Depressing Vitamin D Function*

It was obvious from an examination of the results in Tables 20 and 21 that the depressing effect of the vitamin A doses administered on the

TABLE 22. EFFECT OF A DAILY DOSE OF VITAMIN A (1000 I.U. PER HEAD) ON RESPONSE TO LARGER VITAMIN D SUPPLEMENTS

	I.U. Vit. D Dosed Daily per Head	Av. % Femur Ash		
		No Vit. A Supplement	1000 I.U. Vit. A Dosed Daily per Head	Difference Due to Vit. A Supplement
(20 rats per group)	0.5	39.60	34.63	—4.97**
	1.0	41.90	37.60	—4.30**
	2.0	42.78	40.31	—2.47*
	4.0	43.34	42.21	—1.13
	S.E. for group means ± 0.66			
(18 rats per group)	0.25	35.47	29.82	—5.65**
	1.0	42.32	37.20	—5.12**
	4.0	43.94	40.24	—3.70**
	16.0	43.32	41.29	—2.03*
	S.E. for group means ± 0.59			

TABLE 23. THE RACHITOGENIC EFFECT OF A HIGH INITIAL LIVER STORAGE OF VITAMIN A

Group	Daily Dose per Rat I.U. Vitamin D	Av. % Femur Ash	S. group 1
1 No Vitamin A supplement in pre-weaning stage	0.3	38.64	
2 Dosed vitamin A in pre-weaning stage	0.3	30.42	—8.22**
S.E. for group means ± 0.74 (24 rats per group)			

bone-ash response to vitamin D in these animals was far less than that which had been observed during the course of the earlier work. A trial was therefore conducted to test the possible effect of vitamin A on the bone-ash response to a phosphate supplement. The results shown in Table 24 indicate that vitamin A had a marked effect in depressing both weight gain and percentage bone ash which had been increased by the phosphate supplement.

TABLE 24. THE DEPRESSING EFFECT OF VITAMIN A ON BOTH GROWTH AND BONE-ASH RESPONSE TO A PHOSPHATE SUPPLEMENT

Ration	McCollum 3143	McCollum 3143 + 1% KH_2PO_4			
Group	1	2	3	4	5	6
Vitamin A supplement I.U. per head daily	0	0	30	90	270	810
Av. % femur ash	28.5	36.5	34.2	33.1	29.8	28.8
Av. wt. gain (g.)	27.3	49.3	43.6	41.3	40.6	36.8

S.E. for group means: Femur ash ± 0.94
Wt. gain ± 2.67
(11 rats per group)

DISCUSSION

The data presented in this paper demonstrate that carotene is a rachitogenic factor in green feeds, and since equivalent amounts of carotene and vitamin A have the same rachitogenic potency the factor is more properly vitamin A to which carotene is largely converted in the animal body. The green growing leaves of pasture plants probably contain little or no vitamin D, for in no case has any calcifying effect been observed when extracts from such material have been dosed, and, furthermore, the addition of only a small amount of the vitamin to a green-leaf extract is sufficient to promote an appreciable though depressed degree of bone calcification.

Vitamin D appears to be associated mainly with dead or damaged tissue which, not infrequently, constitutes an appreciable proportion of pasture. From the presence of vitamin D in the dead brown leaves, it seems reasonable to assume that the growing plant synthesizes the provitamin, and the apparently negligible vitamin D content of the green tissue might be accounted for by the suggestion that the density of its pigments is such as to effectively shield the provitamin from solar ultraviolet irradiation. These observations concerning the vitamin D content of plant tissue support the conclusion of other workers (Moore and Thomas, 1948; Moore *et al.*, 1948; Thomas and Moore, 1951), that the amount of vitamin D present in fodder plants varies with the amount of dead foliage exposed to solar irradiation, and the green leaves contain practically none.

In view of the fact that sheep in the central latitudes of New Zealand do not receive sufficient solar ultraviolet irradiation to promote adequate body synthesis of vitamin D (Andrews and Cunningham, 1946), it is not surprising that in this country lambs on winter green feed, which is practically devoid of vitamin D and has a high carotene content, are frequently affected by severe rickets. That a high vitamin A uptake can be a factor responsible for the development of ovine rickets has been demonstrated by the results of a field trial reported elsewhere (Grant, 1954). Of a flock of weaned lambs wintered on a permanent pasture, most of those animals which received an additional supplement of

vitamin A became rachitic while none of the unsupplemented animals developed the disease.

The rachitogenic property of pasture carotene also throws light upon some hitherto inexplicable reports indicating that rachitic animals when turned out to spring or summer pastures may become worse or fail to improve (Akerblom, 1949; Krupski and Almasy, 1936).

From the information gained during the course of this investigation it appears that, for rats on the McCollum 3143 ration, the rachitogenic properties of vitamin A can be represented in the hypothetical Fig. 7.

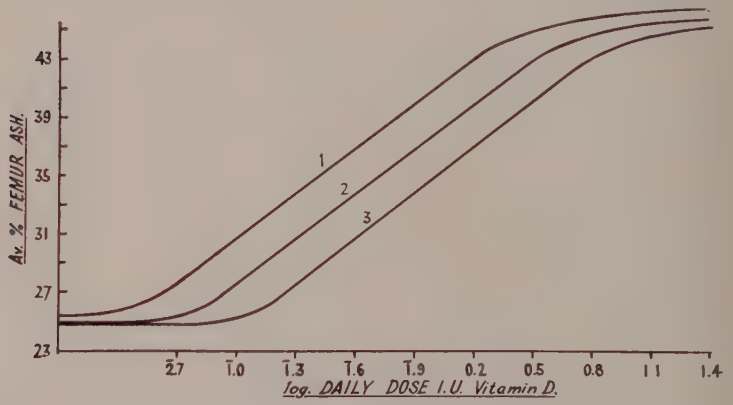

Fig. 7.—Depressing effect of vitamin A on bone-ash response to vitamin D.
1. "Normal" bone ash response to vitamin D.
2. Response depressed by x I.U. vitamin A daily.
3. Response depressed by 10 to 20 x I.U. vitamin A daily.

From the graph it can be seen that in animals receiving small or negligible amounts of vitamin D, the rachitogenic effect of vitamin A is usually not appreciable. The vitamin A depressing effect on bone-ash responses to intermediate vitamin D doses, however, can be considerable. If a vitamin A supplement of x I.U. daily (line 2) halves the normal response to vitamin D, over the greater part of the linear range of the log dose/response regression line, then a daily dose of 10x to 20x I.U. vitamin A will reduce the calcifying effect of vitamin D to one quarter of its normal potency (line 3). The value of x varies widely, probably with the initial vitamin A storage of the test animals, with the carotene content of the diet, and possibly with available phosphorus and other variable constituents of the basal ration. High vitamin A intakes can also depress to some extent bone-ash responses to vitamin D supplements that are considered to be more than adequate.

In some of the trials reported, the daily intake of 1-3 I.U. vitamin A was sufficient to depress appreciably bone-ash response to vitamin D, so that in assaying vitamin D in materials known to be high in carotene or vitamin A an attempt should be made to equalize the vitamin A intake of the animals involved.

While it at first appeared that a quantitative relationship existed between the amount of rachitogenic factor dosed and its depressing effect on the bone-calcifying potency of vitamin D, later experiments showed that this hypothesis was untenable. Whereas the earlier trials indicated that the daily dose of vitamin A or carotene sufficient to halve the bone-calcifying potency of vitamin D was in the region of 5-10 I.U., it was found in later experiments (Tables 20 and 21) that a very much larger intake of vitamin A (500-1000 I.U. daily) was required to produce the same effect.

Some variation in the rachitogenic effect of vitamin A could be explained by a difference in initial storage of vitamin A in the test animals or by differences in content of availability of carotene in batches of the McCollum 3143 ration made up from grain from different sources. It was considered unlikely however that these differences could be sufficient to account for the very large variations observed for the bone-ash-depressing effect of vitamin A, for the test animals were weaned from stock on a standard ration which probably varies but little in its vitamin A content, and it is improbable that the intake of vitamin A of rats on the McCollum ration could vary to any marked degree. It seemed therefore that, instead of vitamin A directly inhibiting vitamin D function, it had an effect on some other factor involved in the calcifying process. Since phosphorus is known to be a limiting factor with regard to the extent of bone calcification in the case of the rat on the McCollum diet, a trial was run (22) to test the effect of vitamin A on the degree of bone calcification and growth rate, both of which had been stimulated by supplementing the basal ration with KH_2PO_4. Vitamin A depressed both the growth and bone-ash response to the phosphate supplement. Since no vitamin D was given to any of the animals in this experiment, and since initial vitamin D storage would probably not be sufficient to affect percentage bone ash differences between groups, the results suggested that vitamin A might have the effect of depressing phosphorus utilization rather than inhibiting the action of vitamin D.

Opposing this view, however, is the fact that in none of the previous experiments did a depression in growth rate result from dosing vitamin A to rats on the McCollum ration, and it is difficult to account for these apparently conflicting results.

The work of Mellanby (1944, 1947) has indicated the importance of vitamin A in regulating osteoblastic and osteoclastic activity, and Fell and Mellanby (1950, 1951, 1952) have indicated that vitamin A is an important factor controlling bone growth and development. It seems possible, therefore, that the rachitogenic property of the vitamin might be attributed solely to its effect at the site of bone formation.

In 1938, Bruce and Phillips tested the effect of carotene on the bone calcifying response to vitamin D in the case of rats on the Steenbock 2965 rachitogenic ration. In one trial these workers found that the degree of calcification assessed by the line test was depressed, but three subsequent experiments of similar design failed to confirm this observation. It is at present not possible to explain these results, and further investigations are being made in this laboratory in the hope that the rachitogenic effect of vitamin A will be more clearly understood. It is clear, however, that, in the aetiology of rickets, the vitamin A status or intake of the animal must be considered with the already complex relationships known to involve calcium, phosphorus, and vitamin D.

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